Genomic and Phenotypic Analyses of Polychaete Sibling Species *Platynereis dumerilii* and *Platynereis massiliensis* in Relation to Ocean Acidification

Thesis

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Genomic and phenotypic analyses of polychaete sibling species *Platynereis dumerilii* and *Platynereis massiliensis* in relation to Ocean Acidification

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Abstract

The increase of anthropogenic carbon dioxide emissions and the subsequent uptake of CO_2 by the sea, is leading to a decrease in the pH of the oceans, a process known as Ocean Acidification. One of the main challenges of the current research on climate change is to determine how marine species respond to low pH/elevated pCO_2 conditions.

This thesis has investigated the effects of natural OA on the polychaete species *Platynereis dumerilii* and its sibling *P. massiliensis* (Annelida, Nereididae) as driver of genetic differentiation and phenotype/genotype selection. *Platynereis* spp. populations were sampled in five geographical areas situated along a thermo-latitudinal gradient along the Italian coasts, characterized by different pH conditions (acid *vs* normal). A multidisciplinary approach, focused on different aspects of the target species biology, was chosen and the following analyses were performed: (a) morphological and morphometric analyses of different populations/genotypes; (b) laboratory rearing of different populations to study the reproductive biology and gamete morphology; (c) population genetics by the amplification of a mitochondrial DNA marker (COI); (d) population genomics by a next-generation sequencing approach (RAD-seq); (e) background analyses and a long term laboratory experiment on selected genotypes/populations to study physiological responses to different pH conditions.

This work has confirmed that *Platynereis dumerilii* and *P. massiliensis* represent two complexes of sibling species characterized by contrasting life history traits, reproductive biology and gamete morphology. The overall *Platynereis massiliensis* predominance in the CO_2 vent systems is not a direct consequence of elevated pCO_2, but it seems to derive from a winning reproductive strategy (brooding habit) in low pH conditions. Unlike *Platynereis dumerilii*, *P. massiliensis* is potentially able to thrive in the CO_2 vents thanks to the higher stability of its antioxidant defence systems over temporal scale and its greater responsiveness to extreme hypercapnia conditions.

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Published papers

Valvassori G, Massa-Gallucci A, Gambi MC (2015). Reappraisal of *Platynereis massiliensis* (Moquin-Tandon) (Annelida, Nereididae), a neglected sibling species of *Platynereis dumerilii* (Audouin & Milne Edwards). *Biol. Mar. Mediterr.*, *22*(1), 113-116

Wäge J, Valvassori G, Hardege JD, Schulze A, Gambi MC (2017) The sibling polychaetes *Platynereis dumerilii* and *Platynereis massiliensis* in the Mediterranean Sea: are phylogeographic patterns related to exposure to ocean acidification?. *Mar. Biol.*, 164:199. doi: 10.1007/s00227-017-3222-x

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Chapter 1

General introduction

1.1 Global Climate Change (GCC)

1.1.1 Ocean chemistry and current knowledge of Ocean Acidification (OA)

The Earth and its biodiversity are accustomed to climatic conditions that vary on daily, seasonal and inter-annual time-scales as highlighted by deep time paleontological records. Accumulating evidence suggests that, in addition to the natural variation, the average climatic conditions measured over extended periods are changing at an unprecedented rate. This unnatural change is known as Global Climate Change (GCC) and can be defined as 'a change of climate which is attributed directly or indirectly to human activity that alters the composition of the global atmosphere and which is, in addition to natural climate variability, observed over comparable time periods' (UNFCCC 1992). Anthropogenic activities have always had an influence on terrestrial and aquatic environments. Since the beginning of the Industrial Revolution, around 1760, this influence has progressively increased due to the increased emissions of greenhouse gasses (GHG) into the atmosphere, such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). These gases are of concern as they alter the energy budget of the earth by changing the net balance of incoming solar radiation and outgoing infrared radiation between the troposphere and the stratosphere (change called 'radiative forcing'). The amount of energy leaving the atmosphere is shrinking, leading to a warming effect with an increment of the ocean temperature (Roemmich 1992), changes in the thermohaline circulation (Roether et al. 1996, Bryden et al. 2005), ice melting (Curran et al. 2003), ocean acidification (OA) and variation in ocean salinity (IPCC 2014).

Among the GHG, CO_2 is of greatest concern due to the large quantity released (IPCC 2014). Atmospheric CO_2 levels are rising because of human activities such as fossil fuel burning and industrial processes, deforestation and other land use change (Raupach et al. 2007). The concentration of CO_2 in the atmosphere has risen from 280 ppm of the preindustrial age, to the modern 380 ppm recorded in 2010 (Feely et al. 2004). Predictions confirm that the CO_2 level in the atmosphere is expected to rise to 800 ppm by the end of the century (Feely et al. 2004, Raven et al. 2005). Carbon dioxide, like other gases, obeys Henry's law, which means that higher levels of atmospheric CO_2 increase the concentration of CO_2 in the ocean surface (Raven et al.

2005). The dissolved CO_2 in seawater exists in four inorganic forms, commonly known as dissolved inorganic carbon (DIC) (Fig. 1.1):

1) Aqueous CO_2;
2) Carbonic acid H_2CO_3;
3) Bicarbonate ion HCO^{3-};
4) Carbonate ion CO_3^{2-}.

$$CO_2\ (g) \leftrightarrow CO_2\ (aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO^{3-} \leftrightarrow 2H^+ + CO_3^{2-}$$

CO_2 reacts with water to form H_2CO_3. Carbonic acid dissolves rapidly to form H^+ ions (an acid) and HCO^{3-} (a base). Seawater is naturally saturated with another base, CO_3^{2-} that neutralize the H^+, forming more bicarbonate.

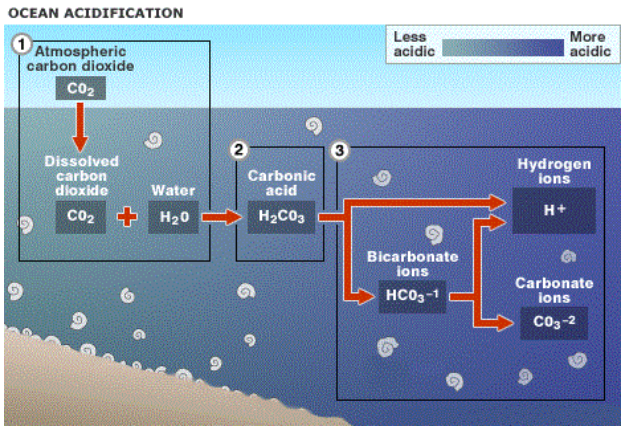

Figure 1.1 The chemical process of ocean acidification.

These chemical properties and the proportion among the different forms of DIC in the seawater enable the ocean to have a role in the seawater pH regulation, acting as a 'carbonate buffer'. Based on this buffering capacity, many scientists initially focused on the benefits of the oceans removing this GHG from the atmosphere. Since the industrial revolution, global oceans have absorbed over 500 billion tons of CO_2, which equates to over one-third of anthropogenic carbon emissions (Sabine and Feely 2007). Decades of ocean observations now show that the downside-ocean uptake of anthropogenic CO_2 is changing the chemistry of the seawater. With increased absorption rates of atmospheric CO_2, the buffering capacity is diminishing, leading to a reaction that reduces the amount of CO_3^{2-} and increases the amount of HCO_3^- and hydrogen ions concentration. The increase in H^+ is translated into a surface seawater pH drop off in a process termed as

'ocean acidification' (OA) (Fig. 1.1). The average pH of ocean surface waters has fallen by about 0.1 units, from 8.2 to 8.1, corresponding to a 26% increase in acidity measured as hydrogen ions concentration (IPCC 2014) (Fig. 1.2). An additional reduction between 0.3 and 0.5 units of pH is expected by the end of the century (Raven et al. 2005, IPCC 2014) (Fig. 1.2), and a reduction of 0.7 units of pH for the 2300 (Caldeira and Wickett 2003).

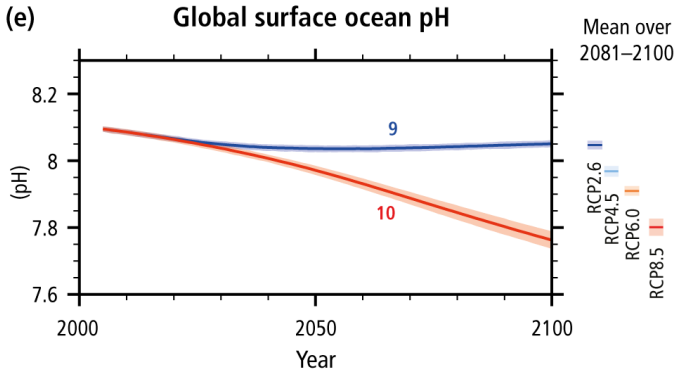

Figure 1.2 Change in ocean surface pH. Time series of projections and a measure of uncertainty (shading) are shown for scenarios RCP2.6 (blue) and RCP8.5 (red). The mean and associated uncertainties averaged over the 2081-2100 period are given for all RCP scenarios as coloured vertical bars on the right hand side (from IPCC 2014, modified).

The altered carbonate chemistry will not only affect the seawater pH. The saturation states of seawater are primarily governed by variations in the ratio of CO_3^{2-} to the solubility product, therefore increased absorption of anthropogenic CO_2 leads to strong changes in calcite and aragonite saturation states (Feely et al. 2004).

In recent decades, the scientific community has started to understand the impacts of the CO_2 rise in the oceans and the first evidence is that the ocean acidification will likely have dramatic consequences for marine biological systems (Munday et al. 2013). Nevertheless, the majority of the ecological effects on the benthic compartment are still unknown (Fabricius et al. 2014).

1.1.2 Impact of Ocean Acidification on benthic biodiversity and the importance of an **in situ** *study approach*

The oceans harbour a huge biological diversity (May and Godfrey 1994). The majority of this marine biodiversity is made up of invertebrates either residing in (infauna) or on (epifauna) sediments, with the benthos containing 98% of all marine species (Snelgrove 1999). Given the importance of the benthic environment as a reservoir for biodiversity, there has been much speculation as to whether ocean acidification has the potential to reduce benthic biodiversity by affecting key biological processes. Kroeker et al. (2013) published a meta-analysis revealing that ocean acidification had an overall significant negative effect (when all taxa were pooled together) on survival, calcification, growth, development and reproduction. This suggests predicted scenarios of ocean acidification will have negative consequences for many marine organisms by the end of this century (Fig. 1.3). Survival and calcification were the two most affected responses by acidification, with 27% reductions in both of them, while growth and development were reduced by approximately 11 and 19%, respectively (Kroeker et al. 2013). The mean abundance was reduced by 15%. Conversely, no relevant effects of acidification on photosynthesis and metabolism were detected when all taxa were pooled together (Kroeker et al. 2013). The photosynthetic activity varied a little among taxa with the exception of calcified algae, for which photosynthesis was reduced to 28% (on average) (Kroeker et al. 2013).

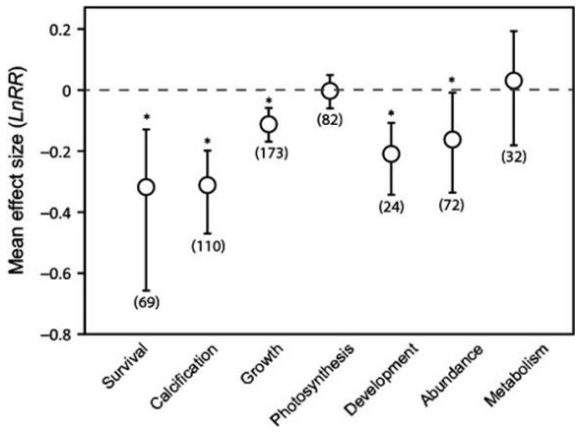

Figure 1.3 Mean effect of near future acidification on major response variables. Significance is determined when the 95% bootstrapped confidence interval does not cross zero. The number of experiments used to calculate the mean is included in parentheses. '*' denotes a significant effect (from Kroeker et al. 2013).

The magnitude of these effects varies among heavily calcified organisms (calcified algae, corals, molluscs, and the larval stages of echinoderms) which are the most negatively impacted, while crustaceans and other non-calcified groups are less affected or even benefit from acidification (Fig. 1.4). However, the authors pointed out that a single taxonomic level responds differently to OA, underlining the importance of species-specific approaches and the need to interpret results with caution without generalizing to the whole marine community (Kroeker et al. 2013).

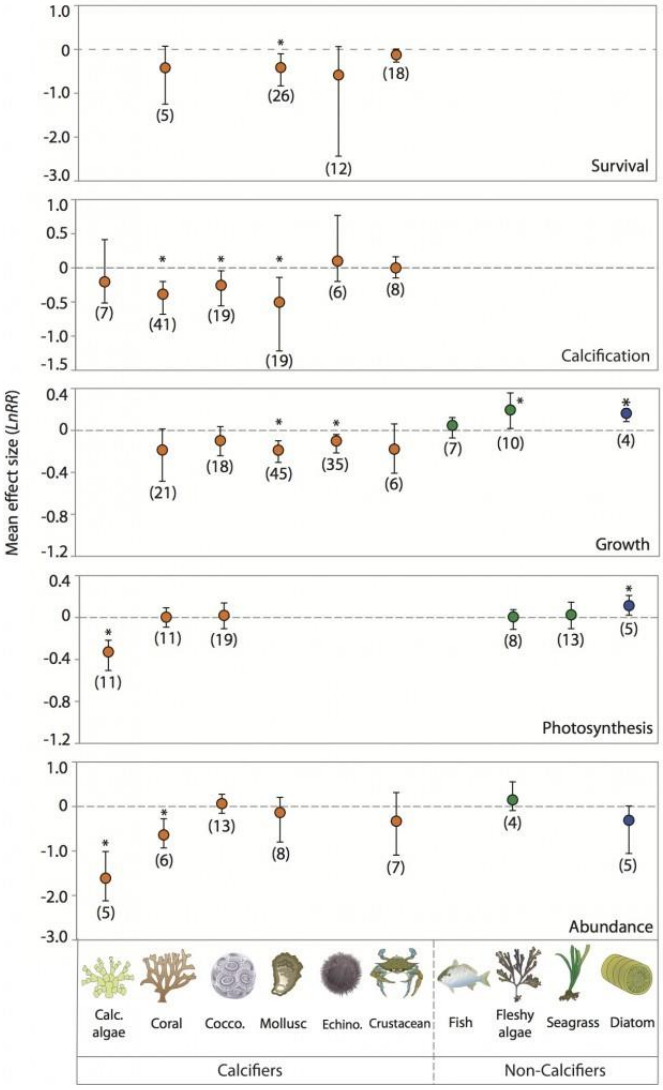

Figure 1.4 Variation in effect sizes among key taxonomic groups, divided by major response variables. Means are from a weighted, random-effects model with bootstrapped bias-corrected 95% confidence intervals. The number of experiments used to calculate the means is given in parentheses. '*' denotes a significant difference from zero (from Kroeker et al. 2013).

The impact of OA has been largely investigated through experiments conducted in laboratory-controlled conditions, mesocosms and prediction models, all mimicking field conditions. Although these approaches have so far led to interesting insights, *in situ* observations have the great advantage of dealing with natural ecosystem responses to low pH/high pCO_2. Among the *in situ* approaches, the ones defined as 'natural experiments' consist of using naturally-altered seawater conditions, such as acidified seawater due to the presence of volcanic CO_2 vent systems (Barry et al. 2010). Although natural experiments have a weak point related to the unpredictability of factors and parameters that may affect the community, they are extremely useful and realistic due to the potential long duration of the treatment. They allow researchers to detect changes caused by chronic exposure to low pH conditions, such as changes in species composition assemblages, evolutionary adaptation processes, etc. (Fabricius et al. 2014, Spicer 2014). In addition, they give the opportunity to study the effects at different hierarchical levels, from single organism to ecosystem. Several studies to assess the effects of exposure to natural low pH conditions, and OA, were performed in different volcanic CO_2 vent systems of the Mediterranean Sea, which have so far been used as 'natural laboratories': the CO_2 vents of Ischia (Naples, Campania) (Hall-Spencer et al. 2008, Kroeker et al. 2011), Vulcano (Calosi et al. 2013a, Vizzini et al. 2013, Milazzo et al. 2014) and Panarea (Sicily) islands (Goffredo et al. 2014).

The CO_2 vents of Ischia are located in a small islet of volcanic origin, called Castello Aragonese. They were the first natural vent systems described and studied (Hall-Spencer et al. 2008), and have been widely utilized in the past 10 years for exploring the effects of low pH on the marine benthic invertebrate compartment (Cigliano et al. 2010, Kroeker et al. 2011, Calosi et al. 2013b, Ricevuto et al. 2014). Studying benthic organism recruitment and the rocky reef benthic community at the Castello Aragonese vent system (Ischia), various investigations highlighted how the invertebrate benthic community richness and composition vary along a pH gradient (Cigliano et al. 2010, Kroeker et al. 2011, Donnarumma et al. 2014, Gambi et al. 2016b). Cigliano et al. (2010) and Donnarumma et al. (2014) clearly showed a biodiversity loss of organisms recruiting in artificial structures along a natural pH gradient, due to reduction of calcifying taxa (Foraminifera, Bivalvia, many Gastropoda and all Spirorbidae polychaetes) as a direct consequence of the low pH conditions on the calcification structure of such organisms.

Kroeker et al. (2011) and Gambi et al. (2016b) obtained similar results. The former authors demonstrated that the invertebrates taxonomic richness undergo a reduction in extreme low pH zones of the Castello vents, primarily due to the disappearance of key calcifying species, such as molluscs and decapods. Conversely, it was found that some small peracarid crustaceans and polychaetes exhibited their highest densities in the extreme low pH zones (Fig. 1.5). These results highlighted a density compensatory effect, but also a reduction in the benthic invertebrate biomass and function in extreme low pH zones. This led to an evident simplification of the trophic structure in the more acidic conditions (Kroeker et al. 2011). One study focusing on polychaetes (Gambi et al. 2016b) confirmed the trends observed for the whole community and highlighted the most robust worm species able to thrive under severe OA conditions.

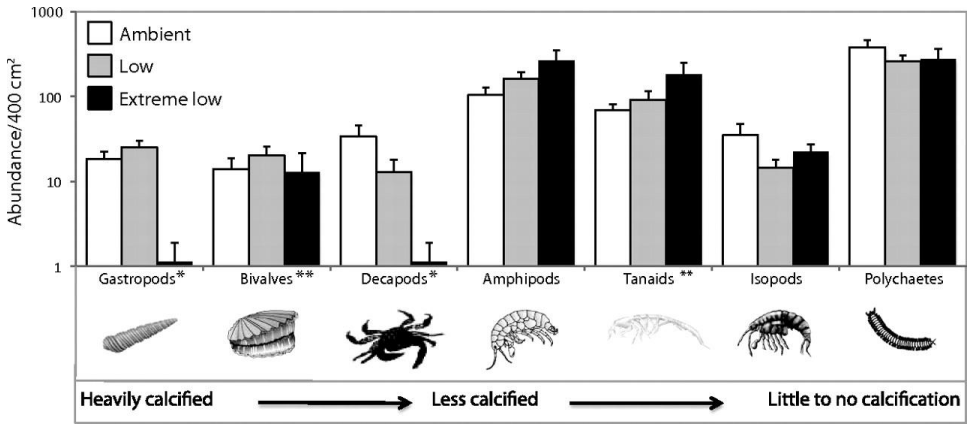

Figure 1.5 Mean densities of individuals within the most abundant taxonomic classes in each pH zone. Taxonomic classes are organized from the most heavily calcified on the left to the least calcified on the right. '*' significant difference in densities between pH zones ($\alpha = 0.05$); '**' a significant interaction (site x pH, $\alpha = 0.05$) (from Kroeker et al. 2011).

Disappearance of calcifiers might not be a direct consequence of the effect of decreasing pH on calcification rates, but it could derive by an impact at the level of organism physiological processes. Pörtner's study (2008) highlighted that calcifiers are likely sensitive to the hypercapnia not because they are calcifiers but because they are sessile, hypometabolic organisms without good capacity to regulate their systemic acid-base status and extracellular pH. Discern between species that are able to resist to pH changes, and those that are not, is one of the major concerns of global climate change (Munday et al. 2013).

As shown by Calosi et al. (2013b), marine organisms may be able to respond via acclimatization (adjusting their physiology, morphology, or behaviour, via phenotypic plasticity) or via adaptation (i.e. selection of genotypes associated with phenotypes better able to cope with elevated pCO_2), to maintain fitness in acidified conditions. The metabolic rates of acclimatized and adapted individuals were different once they were removed from low pH conditions. In acclimatized individuals the metabolic rates returned to a 'normal' status while, in the adapted ones more elevated rates were displayed (Calosi et al. 2013b). The rising CO_2 levels are gradually shifting the fitness landscape of the oceans by triggering changes also at the genotypic composition level of marine species and populations, even with little or no phenotypic change associated (Sunday et al. 2014). Significantly different sensitivities to ocean acidification were observed among four strains of the coccolithophore *Emiliana huxleyi* (Langer et al. 2009) and between genetically different lines of the ecologically and economically important Sydney rock oyster *Saccostrea glomerata* (Parker et al. 2011). A local adaptation to different carbonate chemistry conditions was observed in two populations of the purple sea urchins *Strongylocentrotus purpuratus* from the Northeast Pacific (Kelly et al. 2013). Ocean acidification poses a global threat to biodiversity affecting marine species also from the genotypic point of view; future research directions to understand the modulating role of the seawater carbonate chemistry change at the genetic level, within species and populations, is recommended.

1.2 Sibling species

Biodiversity was evaluated for a long time using simple species inventories based on individual morphological analyses by comparison of phenotypic characters, however speciation processes (simply defined as 'processes that leads to the formation of a new species') do not implicate morphological changes. At this point, it is highly probable that the earth's biodiversity is underestimated (Bickford et al. 2007). The belonging to a species based on a mere morphological analysis, completely neglect the existence of those termed as 'sibling species'. Before specifing the meaning of 'sibling species', it is indispensable to know the 'species' concept. The three species concepts currently referred to are: morphological (MSC), phylogenetic (PSC; Mishler and Theriot 2000) and biological (BSC, Mayr 1969). The MSC is useless for sibling species but, both BSC and PSC are relevant. BSC defines species as 'groups of interbreeding natural populations that are reproductively isolated from other such groups' (Mayr 1969). PSC meaning can be simplified as 'the smallest monophyletic group worthy of taxonomic recognition' (Mishler and Theriot 2000). According to Bickford et al. (2007), the combination of reciprocal reproductive compatibility tests with molecular analyses (BSC and PSC) can represent the best strategy to assess the occurrence of sibling species. The term 'sibling species' is used to describe species that are difficult or impossible to distinguish based on morphological characters (Mayr and Ashlock 1991). Most authors regard 'sibling' and 'cryptic' species terms as synonyms (Sáez and Lonzano 2005), whereas others give them a different meaning (Knowlton 1986, Bickford et al. 2007). Bickford et al. (2007) consider two or more species to be 'cryptic' if they are, or have been, classified as a single nominal species because they are at least superficially morphologically indistinguishable. On the other hand, 'sibling species' are defined as 'cryptic sister species; two species that are the closest relative to each other and have not been distinguished from one another taxonomically'; this definition denotes more recent common ancestry than 'cryptic' (Bickford et al. 2007). A third term 'pseudo-cryptic or pseudo-sibling species' has been coined to refer to cryptic species, once revealed by information such as molecular data, which also show morphological differences or other diagnosable characters, but the boundaries are not clear (Sáez and Lonzano 2005, Nygren 2014). In this PhD thesis work, sibling and cryptic species were used as synonyms.

Even if historically the discovery of new sibling species was made possible by life history traits comparisons, by finer morphological observations and by reproductive biology investigations, it is with the advent of the molecular approaches and the consequent increased availability of DNA sequences that this research branch has increased exponentially (Fig. 1.6) (Bickford et al. 2007). However, according to several authors, an integrated approach using as much data as possible to infer species boundaries, including molecular data, morphology, reproductive biology, ecology and physiology, appears to be one of the most highly informative strategies to follow.

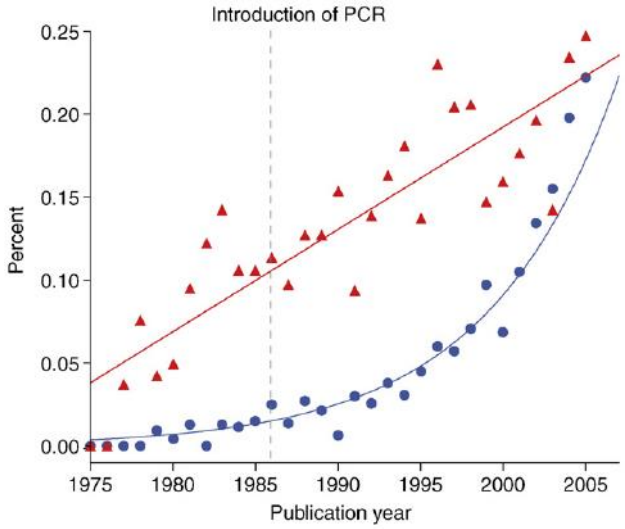

Figure 1.6 Increased recognition of cryptic species.The percent of peer-reviewed publications in Zoological Record Plus (CSA) that mention 'cryptic specie' (circles) or 'sibling species' (triangles) in the title, abstract, or keywords has increased dramatically since the advent of PCR (from Bickford et al. 2007).

The appropriate species identification and recognition of siblings has important implications in several research lines (Bickford et al. 2007, Nygren 2014). Ignoring sibling species most of all leads to an underestimation of the species richness. It may have serious negative effects if it involves species important for medical and economical purposes, with implications in the aquaculture industry (e.g. fisheries management and pest control) and in the conservation, protection and management of natural resources. This exclusion could also generate confusion and alter the results of bioassay for species used as bioindicator organisms in environmental monitoring or eco-toxicological studies (Bickford et al. 2007, Nygren 2014).

Sibling species are common in marine habitats and are found in all major taxonomic groups (Knowlton 1993). Polychaetes represent one of the most dominant taxa in the marine communities and sibling species constitute a large part of their often neglected biodiversity, as proved by several scientific studies focused on the study of model organisms belonging to this class (Nygren 2014). For example, Grassle and Grassle (1976) demonstrated the occurrence of six sibling species in the formerly-believed cosmopolitan polychaete indicator of organic enrichment *Capitella capitata*, revealing only slight morphological differences between species with distinct life history traits and reproductive modes. The cosmopolitan pollution indicator *Polydora ligni* (Annelida, Spionidae) underwent considerable intraspecific divergence with evidence that indicates the occurrence of at least one sibling species (Rice and Simon 1980). The Virgilio et al. (2009) phylogeographic study revealed cryptic diversity in the Nereididae polychaete species *Hediste diversicolor*, largely used in ecotoxicological studies and bioaccumulation assays. Phylogenetic studies on the non-indigenous polychaete *Marenzelleria* in the Baltic Sea revealed the occurrence of at least three different cryptic species with potentially different effects on the indigenous fauna (Blank and Bastrop 2009). Genetic analyses and reproductive biology observations highlighted the occurrence of a neglected sibling of the Evo-Devo and bioindicator model species *Platynereis dumerilii* in relation to ocean acidification conditions (Calosi et al. 2013b, Lucey et al. 2015). The mariculture resource *Perinereis nutia* and the marine bait *Marphysa sanguinea* were both identified as complexes of cryptic species (Glasby and Hsieh 2006, Lewis and Karageorgopoulos 2008). These results were mainly obtained thanks to the use of combined approaches to perform a correct identification of cryptic species, confirming the importance of the application of this strategy of study to unravel and explore the neglected biodiversity of Polychaetes.

1.3 Target species

1.3.1 Platynereis dumerilii*: an important model species*

Platynereis dumerilii (Audouin and Milne-Edwards, 1834) is an annelid worm belonging to the monophyletic family Nereididae. As with any annelid, its body is characterized by rings or segments (Viéitez et al. 2004). This polychaete is a temporary tube-dwelling species that can swim with a characteristic undulating body movement and, in the non-reproductive stage, it spends most of its lifetime in a self-spun mucous tube open at both ends (Daly 1973). It is a meso-herbivore species widely distributed throughout the Mediterranean Sea, inhabiting shallow hard bottoms covered by seaweeds, mainly brown algae (Giangrande 1988, Gambi et al. 2000), or *Posidonia oceanica* and *Cymodocea nodosa* meadows (Scipione et al. 1996). It also inhabits many other parts of the world to the point that it has been defined as 'a cosmopolitan form with wide geographical distribution in warm seas'. Widely studied from the reproductive point of view (Hauenschild 1951, Fischer and Dorresteijn 2004, Fischer et al. 2010), *Platynereis dumerilii* is dioecious/gonochorostic species (separate sexes) characterized by a semelparous life cycle (single reproductive event in its life cycle) with a brief pelagic development (Fischer et al. 2010). In the Mediterranean Sea it reproduces between May and September (Giangrande 1989, Giangrande et al. 2002). During reproduction, both male and female specimens experience a strong morphological transformation, termed 'sexual metamorphosis', into pelagic sexually-mature epitokous form bearing gametes (a stage called 'heteronereis'). Synchronized by lunar periodicity (Zantke et al. 2013) and attracted to each other by pheromones (Zeeck et al. 1988, 1998), the epitokous forms swarm at night and the egg fertilization takes place in the water column with the formation of planktonic larval stages (Fischer et al. 2010). After the spawning event, both male and female die (Fischer et al. 2010).

In recent decades, the annelid *Platynereis dumerilii* has emerged as a well-suited Evo-Devo model species (Tessmar-Raible and Arendt 2003, Raible et al. 2005, Simakov et al. 2012, Zantke et al. 2014), a model organism for the study of molecular developmental processes, evolution, neurobiology and marine biology in various laboratories.

'Classical' and well-established bilaterian animal models belong to the ecdysozoans (fruit fly, *C. elegans*) or deuterostomes (mouse, chicken, fish); conversely, lophotrochozoans (annelids, molluscs, and other marine invertebrates) are still largely under-represented despite their obvious relevance to comparative approaches. *Platynereis dumerilii* is especially suitable for comparative studies as it is considered a slow-evolving species. It has highly conserved cell types and a gene structure with protein sequences, as well as the number and position of introns in its genome, showing lower divergence from deuterostomes (and in particular from vertebrates) than that of other protostomes considered faster-evolving species (Raible at al. 2005). At the cellular level, it possesses a shared common ancestry with other vertebrates and invertebrates in several cell types of the photosensory system, neurosecretory system and mushroom bodies (Arendt et al. 2002, 2004, Tessmar-Raible et al. 2007, Tomer et al. 2010). The gene expression during the development of the two-celled larval eyes may reflect the bilaterian ground pattern (Arendt et al. 2002). The patterning of its central nervous system development shares many similarities with the molecular events which take place in the vertebrate central nervous system development (Denes et al. 2007, Kulakova et al. 2007, Kerner et al. 2009, Steinmetz et al. 2011, Demilly et al. 2013). Molecular research on *Platynereis* segmentation has revealed similar patterning molecules to those used in insect segment formation (Dray et al. 2010). In 1875, Anton Dohrn proposed the 'annelid theory' suggesting that vertebrates originate from annelid-like ancestors. This theory drew its main support from comparative anatomy and it started with a worm that inverted the body on the way to evolving into a vertebrate. In the annelid worms the nerve chord runs along the body in a position which is ventral in relation to (i.e. below) the esophagus or alimentary canal; since in vertebrates the position is reversed (the esophagus is ventral) a vertebrate can be seen as an annelid walking on its back (Dohrn 1875). Currently multiple strings of evidence converge to confirm that *Platynereis dumerilii* represents a good reference species for comparative and evolutionary developmental biology. The great advantage of *Platynereis dumerilii* is that it can be easily bred in captivity and it produces offspring throughout the year (synchronized event) with transparent eggs, embryos and larvae, thus accessible by conventional light microscopy as well as by confocal laser-scanning microscopy (Fischer et al. 2010).

Platynereis dumerilii has also emerged as a polluted waters biological detector. Bellan (1980) established an 'annelid pollution index', which is based on the premise of sensitive modification of the polychaete population as a whole, in order to characterize polluted/disturbed communities *versus* non-polluted/undisturbed ones in the French Mediterranean. This index is based on the ratio of pollution indicators, those species found to dominate in polluted areas, to the indicators of non-pollution, those species found to dominate in the cleaner areas. *Platynereis dumerilii* was one of those species classified as sentinels of organically polluted waters. The ratio of the sums of the dominances of the two species groups furnish the annelid pollution index value that is considered to be a biological measure of the environmental quality. Any index value greater that 1 implies polluted conditions, while if it is inferior to 1 it is in pure or slightly polluted waters (Bellan 1980). There are also reports of *Platynereis dumerilii* used as a model organism in environmental monitoring of chemical contaminants (Bryan and Gibbs 1980, Hutchinson et al. 1995) as well as in ecotoxicology (Lee and Nicol 1981, Ricevuto et al. 2015a). *Platynereis dumerilii* was also classified as a tolerant species to low pH/elevated pCO_2 conditions since it is one of the dominant species in the most intense venting area of the natural vent system of Castello Aragonese in Ischia Island (Italy) (Cigliano et al. 2010, Calosi et al. 2013b, Ricevuto et al. 2012, 2014). *Platynereis dumerilii* can be considered as an efficient sentinel species for different environmental stress conditions (pollution, ocean acidification etc.).

1.3.2 Platynereis massiliensis*: the neglected sibling species of* P. dumerilii

The Nereididae *Platynereis massiliensis* (Moquin-Tandon, 1869) is the only known sibling species of *P. dumerilii*. Recognized as a distinct species also by Hauenschild (1951), *Platynereis massiliensis* (formerly called 'nereidogenic form of *Nereis dumerilii*') is the simpatrically-distributed closest relative of *P. dumerilii*. Compared to *Platynereis dumerilii*, *P. massiliensis* is morphologically indistinguishable at the sexually immature adult stage but has a different reproductive mode from its sibling congener with strongly different reproductive habits, morphologically different gamete and differences in early development (Hauenschild, 1951, Schneider et al. 1992, Lücht and Pfannenstiel 1989). *Platynereis massiliensis* is a protandric hermaphrodite species

with a brooding reproductive strategy. It is characterized by a semi-direct development with lecithotrophic larvae, while lacking epitokous transformation and swimming larval stages (Hauenschild 1951, Schneider et al. 1992). In the Mediterranean Sea, *Platynereis massiliensis* was first described in the Marseille region (type locality) with a poor and incomplete morphological description and with no figures. The species was then reported in the Mediterranean only two more times: in the Gulf of Naples by Hauenschild (1951) and in Banyuls Sur Mer by Schneider et al. (1992). Hauenschild (1951) attempted also a morphological comparison of the proboscis and of the sperm structure with the sibling *Platynereis dumerilii*. Considering that these studies were focused on embryology and larval development and that Hauenschild's work (1951) was in German, these records were not taken into account in taxonomical/ecological investigations (Valvassori et al. 2015). Ecological and monitoring surveys, generally based on the morphological analysis of preserved adults and non-reproductive specimens, recorded only the presence of *Platynereis dumerilii*. Consequently, it is highly probable that the two species were confused for a long time to the point that *Platynereis massiliensis* was neglected and it was not even reported in Mediterranean polychaete checklists and revisions (Arvanitidis 2000, Viéitez et al. 2004, Çinar et al. 2014, Mikac 2015), including those considering the Italian coasts (Castelli et al. 2008, Mikac 2015). Only recently *Platynereis massiliensis* returned under the spotlight (Valvassori et al. 2015). In a detailed study focusing on its reproductive biology and development, the brooding sibling was proposed as a model species for comparative evolutionary developmental studies to investigate the evolutionary conservation and divergence of genetic pathways involved in developmental processes (Helm et al. 2014).

1.4 Background of this research

Sibling species with identical adult morphology but different reproductive biology, gamete morphology and embryogenesis, are frequent among polychaetes (Grassle and Grassle 1976, Rice and Simon 1980, Wilson 1983, Pfannenstiel et al. 1987, Manchenko and Radashevsky 1993, 2002, Sato 1999, Kruse and Reise 2003, Kruse et al. 2003, Sato and Nakashima 2003, Kikuchi and Yasuda 2006, Lewis and Karageorgopoulos 2008, Paxton and Åkesson 2010). The case of the polychaete model species *Platynereis dumerilii* and its sibling *P. massiliensis* is a blatant example. Due to their morphological similarity at the sexually immature adult stage and the scarce literature for the sibling *Platynereis massiliensis*, the two species were confused for a long time and the existence of *P. massiliensis* was ignored even in the Mediterranean polychaete checklists and revisions (Valvassori et al. 2015). The sibling remained practically neglected until Calosi et al. (2013b) published a paper focusing on ecophysiological investigations in the unique naturally acidified waters of the Castello Aragonese CO_2 vent system of Ischia Island (Gulf of Naples, Italy). Studying acclimatization and adaptation processes to acidified conditions, the authors focused on six different polychaete species, among which *Platynereis dumerilii*. The high tolerance of this model organism to ocean acidification was previously highlighted in consideration of its great abundance in acidified low pH areas when compared with normal pH conditions (Ricevuto et al. 2012, 2014). *Platynereis* specimens collected in the most intense venting area of the south side of the Castello (station defined as S3), appeared to be able to physiologically adapt to chronically elevated levels of pCO_2 with increased metabolic rates and lower mean body size (80% lower) when compared to the specimens from control (normal pH) conditions. Then, genetic preliminary results based on the COI marker analysis, showed the selection of a genotype collected in acidified conditions that clustered separately from what we could consider the actual *Platynereis dumerilii* (Calosi et al. 2013b). The COI phylogenetic tree demonstrated that *Platynereis* spp. represents a complex of sibling species and the vent population belongs to a unique genetic clade, which is distinct from the mitochondrial genome of *P. dumerilii* as well as from other samples collected in various control areas outside the vents, in other locations of Italy and of the Atlantic Ocean (Fig. 1.7). Calosi et al. (2013b) asserted that

Platynereis dumerilii was able to physiologically and genetically adapt to chronic and elevated levels of pCO_2.

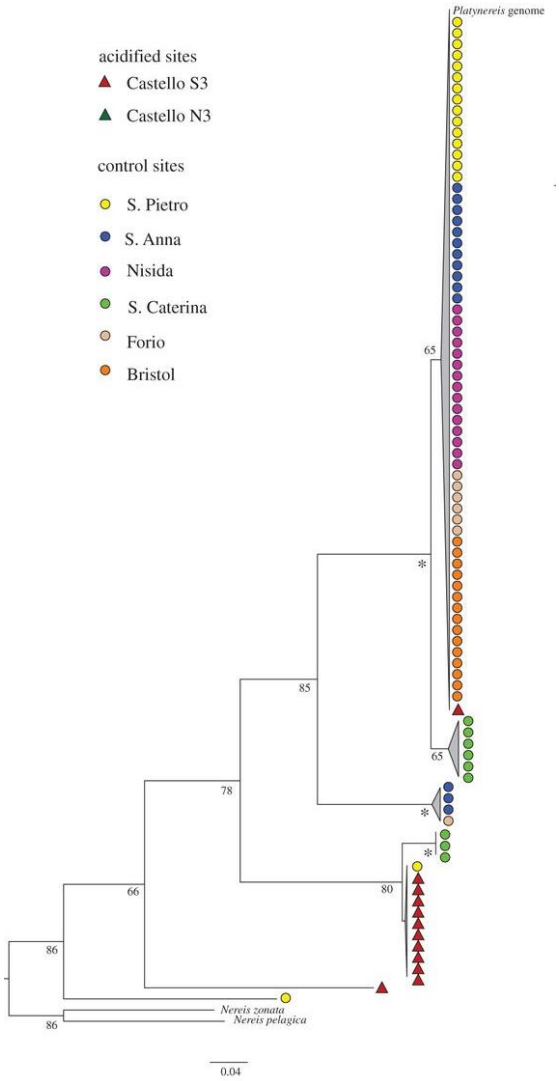

Figure 1.7 Phylogenetic tree resulting from maximum-likelihood analysis of cytochrome *c* oxidase subunit I (COI) data. Branch support is indicated as bootstrap percentages (1,000 pseudoreplicates); asterisk (*) indicates bootstrap value grater that 98% (from Calosi et al. 2013b, modified).

Subsequently, reproductive biology observations in laboratory conditions of specimens collected in the same CO_2 vent area off the Castello, highlighted a brooding reproductive mode previously described for the sibling *Platynereis massiliensis*, instead of the typical *P. dumerilii* broadcasting behavior with the epitokous transformation (Lucey et al. 2015). A cross-breeding experiment between an epitokous male individual from control population (normal pH) and an adult collected within the vent site, resulted in eggs deposition by the vent individual likely due to the occurrence of pheromone-

induced response. Nevertheless, this hybridization was translated into fertilization but not into developed eggs (Lucey et al. 2015). These results validated the identification of *Platynereis massiliensis* as the sibling species inhabiting the vent zone. The vent site of Ischia Island was dominated by brooding *Platynereis massiliensis* (10:1 with *P. dumerilii*), while the control site by the broadcasting species *P. dumerilii* (15:1 with *P. massiliensis*) (Lucey et al. 2015). Cryptic speciation may have been sympatric in the past (in the same habitat), but the distribution of the brooding *Platynereis massiliensis* clearly showed how this species favours high CO_2 habitat, whereas the sibling broadcasting *P. dumerilii* does not (Lucey et al. 2015). This pattern can be interpreted as a solid example of brooding pH-driven factor (Calosi et al. 2013a). The polychaete-based analysis of Lucey et al. (2015), in fact provided evidence that long-term survival of marine species in acidic conditions is related to life history strategies where eggs are kept in protected parental environments (brooding chambers, tubes, jelly etc.) or larvae have no free swimming phases (direct developers).

1.5 Study areas

The present PhD thesis was based on the study of *Platynereis* spp. populations collected from five different study areas, located along a thermo-latitudinal gradient along the Italian coasts (Fig. 1.8). In some study areas, the sampling sites are characterized by different pH conditions. The following study areas were selected in order to obtain an overview of the Italian coasts of the Mediterranean Sea:

- La Spezia, Liguria (North-Western Italy) – an area subjected to a relatively cold water regime with normal pH conditions;
- Ischia Island (Naples), Campania (Tyrrhenian Sea) – low and normal/control pH conditions;
- Panarea Island (Messina), Sicily (Tyrrhenian Sea) – low pH conditions;
- Vulcano Island (Messina), Sicily (Tyrrhenian Sea) – low and normal/control pH conditions;
- Santa Caterina (Lecce), Apulia (Ionian Sea, Eastern Mediterranean) – an area subjected to a warmer water regime with normal pH conditions.

Figure 1.8 Study areas considered in this PhD thesis located along a thermo-latitudinal gradient along the Italian coasts (white spots).

1.5.1. Blue Bay, La Spezia (Ligurian Sea, Western Mediterranean)

The sampling site is close to the city of La Spezia and it is called 'Blue Bay' (Ligurian Sea). It is a small pocket beach just below the ENEA Center at Santa Teresa (44°4'58.08" N, 9°53'2.52" E) (Fig. 1.9). The rocks that surround the beach host a reach algal community, mainly dominated by *Halopteris scoparia* and *Dictyota* spp. The sampling site is located on shallow hard bottoms at about 1-2 m depth.

Figure 1.9 Blue Bay (La Spezia, Ligurian Sea, Western Mediterranean) sampling site (white spot).

1.5.2 Castello Aragonese and Sant'Anna rocks, Ischia Island (Tyrrhenian Sea, Campania, Western Mediterranean)

Ischia is a volcanic island that belongs to the Phlegraean Archipelago, in the Gulf of Naples (Italy, Tyrrhenian Sea). Two different sampling sites located on the north-eastern side of the island were selected, based on the different pH conditions: Castello Aragonese as an acidified site (low pH area), Sant'Anna rocks as a control site (normal pH area) (Fig. 1.10).

Figure 1.10 Castello Aragonese and Sant'Anna rocks (Tyrrhenian Sea, Campania, Western Mediterranean) sampling sites (white spots) are both on the Cartaromana Bay in Ischia Island.

Castello Aragonese is a small volcanic islet connected by an artificial bridge to the main island of Ischia. It is designated as a 'marine geosite', a scientifically and environmentally important geological site (Campania Region n. 20, Gambi and Ricevuto 2012). Ischia has a long and well-documented history of volcanism and it is characterized by shallow waters gas venting (Tedesco 1996). The vents occur on the north and south sides of Castello Aragonese and are characterized by gas emissions, composed of 90-95.3% CO_2, 3.2-6.6% N_2, 0.6-0.8% O_2 and 0.2-0.8% CH_4 (no sulphur), emitted at ambient seawater temperature (Hall-Spencer et al. 2008). The site is microtidal (0.30-0.50 m range); salinity (38%) and total alkalinity (2.5 mequiv.kg^{-1}) are homogeneous along survey stations. The annual temperature range is 13-25 °C (Hall-Spencer et al. 2008), although occasional heat-waves can occur during the summer and the temperature can reach values above 28 °C (Garrabou et al. 2009). The south vent site gases were emitted at 1.4 x 10^6 litre day^{-1} along an area of about 3,000 m^2, while on the north side they are released at a rate of about 0.7 x 10^6 litre day^{-1} in an area of about 2,000 m^2 (Hall-Spencer et al. 2008). These gas emissions decrease seawater pH from the normal value of approximately 8.17 to 6.57, running parallel to the rocky shore on the north and south sides of Castello for 300 m (Hall-Spencer et al. 2008, Kroeker et al. 2011).

The different rates of gas emissions have created pH gradients on both the north and south sides of the islet and three stations were identified with different pH-mean conditions (N1, N2, N3 – S1, S2, S3) (Kroeker et al. 2011) (Fig. 1.11). Stations N1 and S1 are designated as control stations with normal pH (8.0 ± 0.1 and 8.1 ± 0.1); stations N2 and S2 are considered intermediate pH areas, characterized by high pH fluctuations and low pH values (|7.8 ± 0.2| and |7.8 ± 0.3|); N3 and S3 are considered as the most acidic stations, with the lowest pH values and the highest variability (|7.2 ± 0.4| and |6.6 ± 0.5|) (Kroeker et al. 2011). The south side is sheltered and has a shallow (0.5 m depth) *Posidonia oceanica* meadow and a rocky reef macroalgae community, mainly composed of *Halopteris scoparia* and *Dictyota* spp. Our Castello Aragonese sampling site is located in the south acidified sites (S2-S3) (40°43'50.80"N, 13°57'47.80"E), where the CO_2 bubbling from the seafloor drives the seawater pH down to values equal to or lower than business-as-usual IPCC projections for 2100 (pH 6.5-7.8) (IPCC 2014), at least in the most intense venting areas as station S3 (Kroeker et al. 2011).

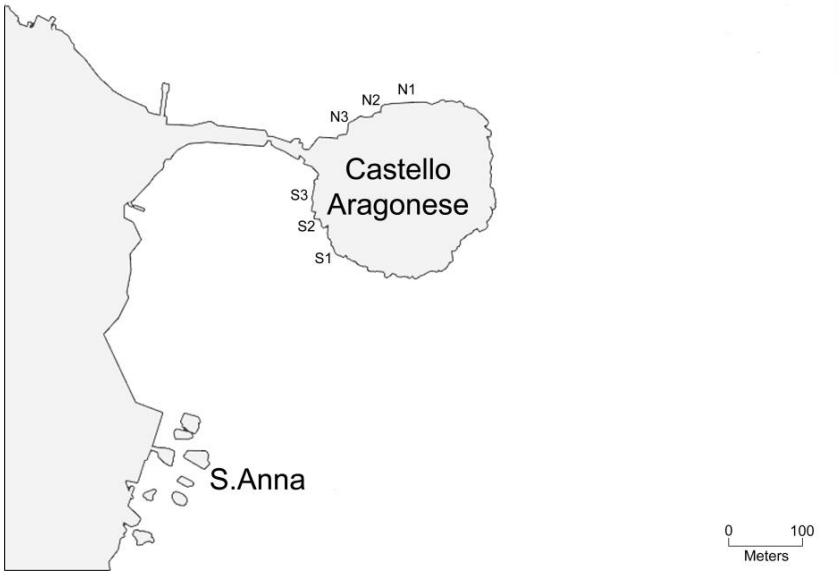

Figure 1.11 Ischia Island sampling sites: Castello Aragonese with acidified (S3, S2, N3, N2) and control (S1, N1) stations and Sant'Anna rocks.

The control zone of our study is an area located 600 m from the south side of the vents, called Sant'Anna rocks (40°43'34.64"N, 13°57'36.33"E) (Fig. 1.10). Mean pH values of Sant'Anna are 8.05 ± 0.001 (late spring) and 8.11 ± 0.001 (fall) (Ricevuto et al. 2015b). The sampling site is characterized by a rocky reef macroalgae community,

mainly composed of *Halopteris scoparia*, *Cladophora prolifera*, *Dyctiota* spp. and *Jania* cfr *rubens* (Ricevuto et al. 2015b).

1.5.3 Ditella beach and Bottaro crater, Panarea Island (Aeolian Archipelago, Sicily, Tyrrhenian Sea)

Panarea is the smallest of seven islands of the Aeolian Archipelago (Southern Tyrrhenian Sea, Sicily). It is characterized by the presence of several diversified CO_2 vent systems of gas and hot/cold water submerged emissions of volcanic origin (Italiano and Nuccio 1991, Gugliandolo et al. 2006). The sampling sites considered are the acidified areas in front of Ditella beach, termed as 'Hot/Cold points' (38°38'21.16"N, 15°4'42.84"E) (Rogelja et al. 2016), and the Bottaro islet crater (38°38'15.73"N, 15°6'36.13"E) (Fig. 1.12). Both stations are located at 10-11 m depth. The Hot/Cold points are characterized by hot waters (up to 60°C) and gas emissions mainly composed of CO_2 (98%) and H_2S (from 0.4 to 4.0%), in addition to very low volumes of other gases like N_2, H_2, He and CH_4 (0.4%). Average pH values vary from 7.9 at the COLD station to 5.6 at the HOT one (Rogelja et al. 2016). A *Posidonia oceanica* meadow and large patches of sandy-gravel sediments, characterize the Ditella Hot/Cold points. Since this area is a mooring zone for boats, there are large moors (concrete and cement) dispersed on the bottom densely covered by the brown alga *Cystoseira foeniculacea*. Over moors, were macroalgae were collected, the heat produced by the hot water emissions is dispersed and it was recorded an ambient seawater temperature. In Bottaro crater, gases are emitted at ambient temperature, they consist of 98-99% CO_2, 0.3-0.3% N_2, 0.01-0.02% O_2, 0.001-0.002% CH_4, 0.3-0.6% H_2S by volume and the CO_2 dissolution creates a pH gradient from 8.1 to 7.7 (Goffredo et al. 2014). The acidified area around Bottaro crater features a dense cover of another large brown Fucales alga, *Cystoseira brachycarpa* var. *balearica* (Gambi et al. 2016a). Specimens of *Platynereis* spp. were collected in association with these two macroalgae of the genus *Cystoseira* at ambient seawater temperature in October 2015.

Figure 1.12 Ditella Hot/Cold points and Bottaro crater (Panarea Island, Aeolian Archipelago, Sicily, Tyrrhenian Sea) sampling sites (white spots).

1.5.4 Levante Bay and Ponente Bay, Vulcano Island (Aeolian Archipelago, Sicily, Tyrrhenian Sea)

Vulcano is the southern-most island of the Aeolian Archipelago (Southern Tyrrhenian Sea, Italy); it is a volcanically active site located approximately 24 km off the north-east coast of Sicily (Vizzini et al. 2013). On this island, two different sampling sites were selected: Levante Bay as an acidified site (low pH area) and Ponente Bay as a control site (normal pH area) (Fig. 1.13).

Figure 1.13 Levante Bay and Ponente Bay (Vulcano Island, Aeolian Archipelago, Sicily, Tyrrhenian Sea) sampling sites (white spots).

The main vent system of the island is documented at less than 1 m depth in Levante Bay, a shallow bay located on the eastern side of the island (Boatta et al. 2013). This underwater venting gas field occurs and extends over a 130 x 35 m area adjacent to a sandy beach in the Isthmus area, with a total bubbling CO_2 output of 3.6 tons/day and a temperature range of 18.6 - 27.7°C (Inguaggiato et al. 2012, Boatta et al. 2013, Johnson et al. 2013). These underwater gas emissions are composed of 97-98% CO_2 and small quantities of H_2S, which rapidly decrease with distance from the gas vents, from 2.2% to 0.005% (Boatta et al. 2013). This is a microtidal region where volcanic CO_2 vent activity creates a seawater pH gradient ranging from 8.2 to 6.8, based on which three different stations were previously identified (Johnson et al. 2013). All stations were at ambient temperature. Station S1 was located outside the vent in an area with normal and relatively stable mean pH (8.18); S2 as an intermediate station with 8.05 as mean pH value; S3 as a low pH station with a mean pH value of 7.49 (Johnson et al. 2013) (Fig. 1.14).

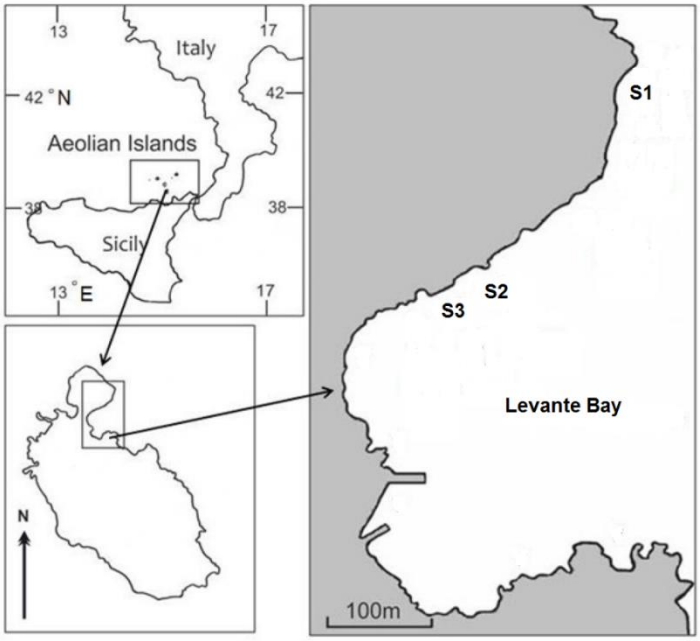

Figure 1.14 Map of the study area, Levante Bay (Vulcano Island), showing sampling stations S1, S2, and S3 (from Johnson et al. 2013, modified).

The water composition is close to that of Mediterranean surface waters in terms of major elements, with the exception of dissolved Fe concentrations that showed a great variability and maximum values close to the vents (Vizzini et al. 2013). Samples were collected in the most acidic station S3 of Levante Bay (38°25.10'37" N, 14°57'41.11" E) (mean pH value measured at sampling time, end May 2015, was 7.19 ± 0.12) collecting macroalgae, such as *Cystoseira compressa* and *Dictyota dichotoma* (Vizzini et al. 2017).

The control area of Vulcano Island is the rocky side of the Ponente Bay beach (38°25'15.52" N, 14°57'17.72" E), far from the vent area, with normal pH conditions (mean pH value measured at sampling time, end May 2015, was 8.1 ± 0.02). It is characterized by sparse rocks and boulders, densely covered by brown macroalgae mainly *Cystoseira compressa* and *Dictyota dichotoma* (Fig. 1.13).

1.5.5 Santa Caterina, Lecce (Apulia, Ionian Sea, Eastern Mediterranean)

Santa Caterina is located in the Ionian Sea, approximately 15 km from Porto Cesareo MPA – Marine Protected Area, towards Gallipoli (Apulia coast). It is a pristine area, characterized by a rocky coastline and algal cover mainly consisting of *Jania rubens*, *Dictyota dichotoma* and *Dictyota* cf *linearis*. The sampling was performed at the entrance of Santa Caterina (40°08'20.7" N, 17°58'47.8" E), on the rocky reef at 0.5-1 m depth in May and September 2015 (Fig. 1.15).

Figure 1.15 Santa Caterina (Lecce, Ionian Sea, Eastern Mediterranean) sampling site (white spot).

1.6 Research aims and objectives

The overall aim of this PhD thesis was to study the two sibling species *Platynereis dumerilii* and *P. massiliensis*, from genomic and phenotypic points of view, evaluating the potential effect of Ocean Acidification (OA) as a driver of genetic differentiation and genotype/phenotype selection. This goal was reached through the study of eight different populations of *Platynereis* spp., sampled in five different geographical areas situated along the Italian coasts in which different pH conditions occur. Some of these populations already shown evidence of genetic differentiation (Calosi et al. 2013b). As previously described in paragraph 1.5 (see above), the selected study and collection areas were: La Spezia, Ischia Island (inside and outside the acidified area), Panarea Island (inside the acidified areas), Vulcano Island (inside and outside the acidified area) and Santa Caterina.

To accomplish the overall aim, a multidisciplinary approach focusing on different aspects of the biology of the model polychaete *Platynereis dumerilii* and its sibling species *P. massiliensis* was chosen, ranging from morphological/morphometric to molecular biology analyses, and from life history and reproductive biology to eco-physiological studies.

The more specific objectives of this thesis can be summarized as follows:

1) To perform a detailed morphological/morphometric analysis of the studied populations, in order to detect and quantify potential differences among *Platynereis dumerilii*, *P. massiliensis* and other putative sibling species, or among populations themselves in relation to ocean acidification conditions (Chapter 2);
2) To detect potential differences in the life history traits, reproductive biology and gamete morphology among the studied populations of *P. dumerilii, P. massiliensis* and putative sibling species, or among populations themselves in relation to ocean acidification conditions (Chapter 3);
3) To study the genetic differentiation of *P. dumerilii* and *P. massiliensis*, and its correlation to ocean acidification conditions, through the genotyping of specimens from each population by the analysis of the mitochondrial DNA marker (cytochrome *c* oxidase subunit I, COI) (Chapter 4);

4) To investigate more thoroughly the genetic relationships among sibling species and different pH conditions (acid *vs* normal), through the use of a next-generation sequencing approach (NGS): the restriction site associated DNA sequencing (RAD-seq) (Chapter 5);
5) To compare antioxidant defence systems responses of *P. dumerilii* and *P. massiliensis* to ocean acidification, through background analyses (wild specimens that came from natural conditions) and after a long-term (30 days) laboratory experiment of exposure to different pH conditions (Chapter 6).

This research will help towards providing a more comprehensive understanding of the overall effect of OA on the genomic and phenotypic selection of the sibling species *Platynereis dumerilii* and *P. massiliensis*. Based on previous studies, the main expected outputs are the identification of further siblings, whose geographic distribution may be influenced by the different native pH conditions. These siblings may be characterized by few morphological/morphometric differences and different reproductive strategies/gamete morphology. Specimens collected in the CO_2 vent systems are expected to exhibit a reduced body size, as consequence of the higher metabolic costs that could occur in low pH/elevated pCO_2 conditions, and a parental care reproductive strategy with direct or semi-direct development of juveniles (Calosi et al. 2013b, Lucey et al. 2015). Furthermore, it is hypothesized a higher efficiency of the antioxidant defence systems of *Platynereis* spp. populations sampled in naturally acidified systems (Calosi et al. 2013b, Ricevuto et al. 2015). Considering the morphological similarity and the wide emplyment of the target species as model organisms, especially *Platynereis dumerilii*, previous records should be reconsidered in light of the 'sibling problem' and the two siblings might be used for comparative studies contributing to a better knowledge of the effects of Global Climate Change on marine biodiversity.

Chapter 2

Morphological and morphometric dissimilarities among *Platynereis* spp. populations from different pH conditions (acidified *vs* normal pH)

2.1 Introduction

Rising atmospheric carbon dioxide (CO_2) concentrations deriving from anthropogenic emissions, represents one of the major drivers of global change and one of the most profound environmental threats to marine biodiversity and ecosystems (Fabry et al. 2008). Since large-scale industrialization, the concentration of CO_2 has dramatically increased driving changes in seawater carbonate chemistry and pH in an ongoing process known as ocean acidification (OA) (Gattuso and Buddemeier 2000, Raven et al. 2005). According to the Intergovernmental Panel on Climate Change, the continued release of CO_2 into the atmosphere is expected to result in a pH reduction of 0.3-0.5 units in the ocean surface water by the end of the current century (IPCC 2014, Caldeira and Wickett 2003, Feely et al. 2004). Research to evaluate the consequences of this phenomenon on marine organisms has greatly increased during the past decades, and an increasing number of *in situ* investigations have been carried out in those environments where water is naturally acidified, as in CO_2 vent systems, defined as 'natural laboratories' for the study of OA (Barry et al. 2010). These unique systems are characterized by the presence of submarine shallow CO_2 emissions of volcanic origin that, due to the elevated partial pressure of CO_2/low pH levels, mimic future possible scenarios of increasing acidification on ecosystem structure (Hall-Spencer et al. 2008).

At the CO_2 vent system of Ischia Island (Gulf of Naples) (Hall-Spencer et al. 2008) the benthic invertebrate settlement ability and communities' structure have been characterized in relation to the pH gradient (Cigliano et al. 2010, Kroeker et al. 2011, Gambi et al. 2016b). Based on distribution patterns, species found inside and/or outside the vents have been defined as 'tolerant' (high density both inside and outside the vents) or 'sensitive' (decreased density or completely absent in the vent system) (Calosi et al. 2013b). The polychaetes, as one of the most abundant taxonomic groups in the vent system of Ischia Island (Kroeker et al. 2011), were selected as model organisms for an *in situ* transplant experiment in the shallow CO_2 vent system of Castello Aragonese in order to investigate the effect of low pH on metabolic rates (Calosi et al. 2013b). A number of tolerant and sensitive non-calcifying polychaete species were used, including specimens morphologically identified as *Platynereis dumerilii* as tolerant organisms. A mutual transplant experiment coupled with preliminary genetic analysis highlighted an overall higher mean metabolic rate and a genetic differentiation of *Platynereis*

individuals collected from the CO_2 vents when compared with the ones from outside. The increased metabolic costs of the vent population have been translated in a strong decrease in the mean body size of the individuals (mean maximum body mass – mg – 80% lower). Calosi et al. (2013b) results suggested that *Platynereis dumerilii* represents a complex of cryptic species with a CO_2 vent strain physiologically and genetically adapted to low pH conditions. Subsequently, the vent population was actually identified as *Platynereis massiliensis* (Moquin-Tandon, 1869), already known as the sibling species of *P. dumerilii* (Hauenschild 1951, Lucey et al. 2015, Valvassori et al. 2015). As reported by previous records, the two sibling species show stronger dissimilarities in the reproductive modes but are morphologically indistinguishable at the sexually immature adult stage, with few differences in the body size and pigmentation (Hauenschild 1951, Schneider et al. 1992, Hartmann-Schröder 1996). *Platynereis massiliensis* was described as a smaller species, which rarely exceed the size of 50 mm in body length, with a more stocky body and a darker pigmentation of *P. dumerilii* (Hauenschild 1951, Hartmann-Schröder 1996).

The species body size is an important feature that must be taken into account in taxonomic invesigations since it can change over time in relation to climate. Experimental evidence indicates that ocean acidification (Jokiel et al. 2008, Ries et al. 2009) and increased temperature can reduce the growth rate and size of various organisms (Daufresne et al. 2009). Warmer and acidified waters negatively affect marine ectotherms causing increased metabolic rates and the consequence reduced body size unless organisms can compensate somehow. The fact that sibling species are defined on the basis of their morphological similarity might lead to assume that all sibling species are difficult to distinguish, but many taxonomic uncertainties stem from the failure to use or consider potentially available characters (Knowlton 1993). In corals, for example, colony growth form has been underutilized as a taxonomic character because of the false assumption that growth form is invariably highly plastic (Knowlton et al. 1992). Even if sibling species have minor morphological differences that are noticed only once species are recognized as distinct for other reasons, in some case these differences are subtle but diagnostic (Knowlton 1993).

Considering the above mentioned possible morphological differences between the two *Platynereis* sibling species, the aims of this study are to: (i) verify whether differences exist in the mean body size of individuals collected from naturally acidified

vent systems and control areas due to the higher metabolic costs that could occur in chronic conditions of low pH (as reported by Calosi et al. 2013b); (ii) evaluate whether different pH conditions, geographical origin and putative reproductive isolation, may reflect morphological differences among sibling species/populations; (iii) make a detailed morphometric analysis on specimens genetically identified as *P. dumerilii* and *P. massiliensis* (see Chapter 4) in order to detect differences at a species-specific level.

According to previous studies, it is expected to detect differences in the *Platynereis* populations mean body size that reflect the effect of the exposure to different pH conditions, especially a shrinking body size of the vent populations. It is supposed to find differences from the morphological and morphometric comparison, at least between the two populations genetically identified as *Platynereis dumerilii* and *P. massiliensis*, even though a taxon with such plasticity in morphology through reproductive mode should be tricky to characterize.

2.2 Materials and methods

2.2.1 Nereididae as an idealized polychaete body plan: the target species **Platynereis dumerilii** *and* **P. massiliensis**

Annelida were originally recognized as a phylum of soft-bodied invertebrate worms by Lamarck (1801). The term 'annelid' comes from the Latin, *annulus*, a little ring, which refers to the typical metameric segmentation of the Annelid's body which is divided into identically organized longitudinal segments (homonymous segmentation) (Giangrande and Gambi 1998). This characteristic organization, also called metamerism, is confined to the trunk region of the organisms (Rouse and Pleijel 2001).

The Polychaete class represents the most diverse group inside the annelid and, according to the metameric segmentation patterns, the polychaete body consists of three fundamental regions: cephalic, trunk and anal. Both cephalic and anal regions have no metameric nature. The cephalic region is split into prostomium and peristomium: the first one is the anteriormost pre-segmental part of the body that contains the cerebral ganglia; whereas the peristomium is the posterior part of the recognizable head that brings projections (Fig. 2.1). The mouth is situated ventrally behind the prostomium. The anal region is the extreme posterior end of the body, called pygidium (Fig. 2.1). New segments are formed sequentially during growth from the anterior edge of the pygidium, so the youngest segment on the polychaete body is always the posterior one. Finally, the trunk region consists of serially repeated segments connected by digestive, vascular, muscular and nervous systems (Rouse and Pleijel 2001) (Fig. 2.1). The term 'polychaete' (derived from two Greek words: *poly*, many, very; and *chaite*, long hair) refers to the occurrence of pairs of laterally placed sets of bristles, called chaetae (or setae), on each body segment. Chaetae are mostly composed of β-chitin bound with tanned (sclerotized) protein and are characterized by an incredible variety of shapes and sizes that make them a useful taxonomic distinctive feature (Rouse and Pleijel 2001).

Among polychaetes, the family Nereididae (Johnston, 1865) is one of the most common and widespread families in marine habitats and has been widely studied. Nereididae are characterized by an eversible pharynx (anterior part of the digestive tract), an axial muscular proboscis which is divided into an outer (maxillary, bearing jaws) and an inner (oral) ring.

The rings are divided into eight smaller areas, referred to as I-IV on the maxillary ring and V-VIII on the oral ring. Both rings become visible when the pharynx is everted. The pharyngeal cavity is characterized by the presence of chitinous denticles, called paragnaths, exposed to the external surface whenever the pharynx is everted. An important diagnostic feature for the taxonomy of this family is the arrangement of paragnaths over both maxillary and oral rings. Another distinctive characteristic of this family is the presence of parapodia, lateral segment appendages: each of them consists of two branches, a dorsal notopodium and a ventral neuropodium, bearing respectively noto- and neuro-chaetae (Fig. 2.1). Organisms belonging to this family are widely employed in Annelid and polychaetes teaching as they represent a classic example of an 'idealized body plan' (Rouse and Pleijel 2001).

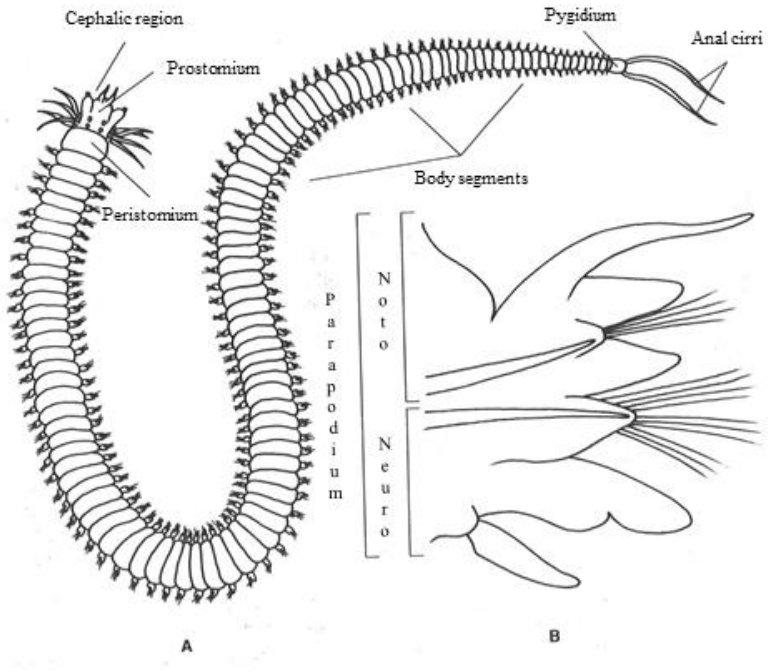

Figure 2.1 Nereididae body (A) and parapodium (B, notopodium and neuropodium bearing noto- and neuro-chaetae) scheme (from Viéitez et al. 2004, modified).

In recent decades, the nereidid *Platynereis dumerilii* (Audouin and Milne-Edwards, 1834) has emerged as an Evo-Devo model (Tessmar-Raible and Arendt 2003, Arendt et al. 2004, Fischer and Dorresteijn 2004, Simakov et al. 2012, Zantke et al. 2014) as well as a pollution indicator species (Bellan 1980).

Considering the wide employment of this model organism in several branches of biology and ecology, it is extremely important to be able to morphologically identify this species. As we worked on Mediterranean specimens, the *Platynereis dumerilii* description of Viéitez et al. (2004) in Fauna Iberica was used for our morphological comparisons. *Platynereis dumerilii* adult individuals are characterized by an anteriorly thickened body, up to 1.5-2 mm in width (excluding parapodia), that are gradually tapered towards the posterior end, for a total body length of about 15-50 mm. The number of segments bearing chaetae of which the body is composed, also called chaetigers, is around 70, and the body colour varies from green to red or brown. The prostomium is sub-piriform and bears sensory structures such as a pair of antennae and palps of comparable length. The peristomium features four pairs of sensory projections, called tentacular cirri. Paragnaths are organized into short combed bars arranged in the areas III and IV of the maxillary ring: in the III area they are arranged in parallel small groups, while in the IV one they form several oblique parallel bars of various length, continuous or discontinuous (Fig. 2.2). There are no paragnaths in the V area of the oral ring; VI area has two short and transverse double pectinated bars; VII-VIII areas have up to five pairs of short bars arranged in two discontinuous rows (Fig. 2.2).

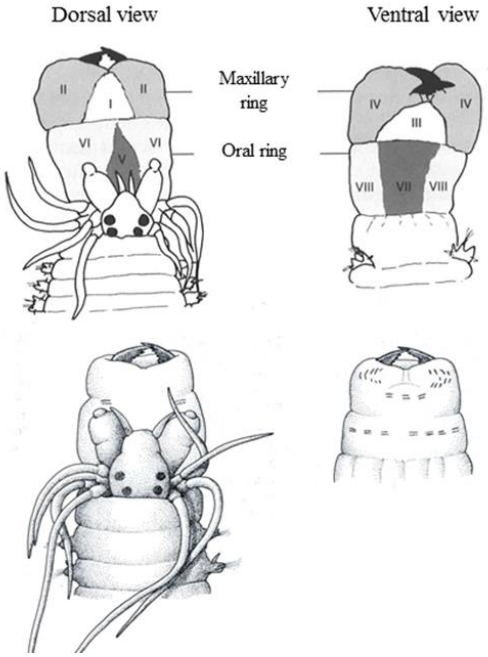

Figure 2.2 Dorsal (left side) and ventral (right side) views of the *Platynereis* spp. pharynx oral and maxillary rings with subareas and paragnaths (from Viéitez et al. 2004, modified).

A further diagnostic feature is the presence of the typical homogomph falciger notochaeta: a distally blunt and curved chaeta of the notopodium (i.e. dorsal branch of segmentally arranged projections that acts as real feet) with articulation distinctly and symmetrically at a right angle to the long axis of the shaft (Fig. 2.3). The homogomph falciger notochaetae are located in the posterior half of the worm body, starting from the 16-20th chaetiger along the antero-posterior axis, at a number of 2-3 per chaetiger. In juvenile specimens, of about 15 chaetigers, these chaetae may appear from the sixth chaetiger (Viéitez et al. 2004).

Figure 2.3 *Platynereis dumerilii* homogomph falciger notochaeta: a distally blunt and curved chaeta of the notopodium with articulation distinctly and symmetrically at a right angle to the long axis of the shaft (from Viéitez et al. 2004, modified).

Platynereis massiliensis (Moquin-Tandon, 1869) is the only known sibling species of *P. dumerilii*. Nowadays, there are only two scarce morphological descriptions of this species reporting almost no morphological differences at the sexually immature adult stage compared to the sibling *Platynereis dumerilii*. As described by Hartmann-Schröder (1996), *Platynereis massiliensis* specimens rarely exceed the size of 50 mm in body length, compared to *P. dumerilii* adult specimens which can reach a length of 100 mm. Conversely, Hauenschild (1951) reported only some morphological differences in the body thickness and pigmentation. *Platynereis dumerilii* was described with a thinner body and pale pigmentation, whereas the author highlighted a more stocky body and a darker pigmentation in *P. massiliensis*.

2.2.2 Sample collection and processing

Platynereis spp. specimens for morphological analysis were collected through a qualitative sampling protocol in four geographical location (six sampling sites), characterized by different pH conditions and situated along a thermo-latitudinal gradient along the Italian coasts: Castello Aragonese (Ischia Island) and Levante Bay (Vulcano Island) as naturally acidified sites; Blue Bay (La Spezia), Sant'Anna (Ischia Island), Ponente Bay (Vulcano Island) and Santa Caterina (Lecce) as normal pH sites. Between April and May 2015-2016, macroalgal thalli, mainly belonging to the species *Halopteris scoparia*, *Cystoseira* spp. and *Dictyota* spp., were collected by SCUBA diving or snorkelling (approx. at 0.5-2.5 m depth). They were placed inside cotton fabric bags (25 x 25 cm, approx. 500 gr w.w. each) and then transported to the laboratory. Finally, *Platynereis* spp. specimens, recognisable by the typical sinuous swimming behaviour, were sorted by macroalgae through visual identification and temporarely stored in Petri dishes (200 ml bowl approx. 30-40 worms each). To maximise the sample collection, trays with macroalgae and seawater were left covered overnight (approx. 10 hours) with a plastic lid to induce slight low-oxygen conditions. The following day, residual *Platynereis* specimens were collected at the water/air interface where they moved to get oxygen. After collection, all worms were placed in an anaesthetic solution of 75% magnesium chloride ($MgCl_2$) for few minutes. Once the animals were completely relaxed, they were fixed in 4% buffered formalin for 24 hours, then washed and stored in 70% ethanol for morphological analysis at the stereo and optical microscopes.

2.2.3 Data analysis

Platynereis spp. samples were analysed from both morphological and morphometric points of view using the stereo microscope Leica MZ125 and the optical microscope LeitzDialux 20 EB. Biometric analysis and relationships were performed on animals collected with quantitative sampling by Kroeker et al. (see methods Kroeker et al. 2011) following a pH gradient in the submarine CO_2 vents off Castello Aragonese and used as reference. A sub-sample of 30 *Platynereis* specimens, corresponding to the largest size range available (from the smallest to the largest individual), were selected with the aim of finding the best parameter to represent the size of the animals (biomass) through

biomass/allometric relationships. For each specimen weight (biomass), body length (excluding anterior tentacular cirri and posterior pygidial cirri), body width (measured at the level of the 2nd anterior chaetiger, excluding parapodia) and number of body segments were measured (Fig. 2.4). The body length has been identified as the best parameter to represent the biomass of the species (see paragraph 2.3 Results, Fig. 2.6a) and it was used to estimate the mean body size of the studied populations. In case of incomplete animals, the body width was used as an alternative proxy (Fig. 2.6b). The analysis was performed on 56 specimens from the acidified area of Castello Aragonese, 86 specimens from Sant'Anna, 58 specimens from the CO_2 vent system of Levante Bay, 16 specimens from Ponente Bay, 53 specimens from Blue Bay and 55 specimens from Santa Caterina. The biometric relationship obtained by Kroeker et al. (2011) samples was used to calculate the population body biomasses (w.w.) from the measures of body length/width. The mean body masses of the studied populations were than compared with the Kruskal-Wallis nonparametric test and the post hoc Dunn's multiple comparison test, with Bonferroni method, thanks to RStudio software (RStudio team 2015).

The morphological comparison among populations was performed based on the parapodia shape, the homogomph falciger notochaetae shape, and the arrangement of paragnaths over both maxillary and oral rings. The parapodia considered were at the IV and at the XIII chaetigers from the prostomium and at the 10th body segment from the pygidium. The observed homogomph falciger notochaetae were the first one from the prostomium and a posterior one near the pygidium.

Based on the population genetic results (see Results – Chapter 4) 20 specimens from both Castello Aragonese and Blue Bay, respectively identified as *Platynereis massiliensis* and *P. dumerilii*, were employed for a detailed morphometric analysis to verify the occurrence of differences between the two siblings. The selected specimens had comparable body sizes (body length and body width) in order to avoid the detection of morphometric differences between the siblings due to different allometric relationships. The two species/populations were compared by measuring body length, body width, number of chaetigers, dorsal cirrus length, dorsal cirrus width and length of the superior lobe (at the level of the IV, XIII chaetigers from the prostomium and the 10th body segment from the pygidium) (as shown in Fig. 2.5), the first homogomph falciger notochaeta position (from the prostomium), blade length of the first and a

posterior homogomph notochaeta. These measurements were obtained through the image software analysis National Instruments IMAQ Vision Builder 6. Data were analysed with the permutational multivariate analysis of variance (PERMANOVA, square root transformation and Euclidean distance), the multidimensional scaling analysis (MDS, square root transformation and Euclidean distance), ANOSIM (factor: species) and SIMPER (factor: species and Bray Curtis similarity) in Primer v 6 (Clarke and Gorley 2006).

Figure 2.4 Graphic measurement scheme of *Platynereis* spp.: yellow line identifies body length (keeping out anterior tentacular cirri and posterior pygidial cirri); blue line identifies body width (measured at the level of the 2nd anterior chaetiger, excluding parapodia); green boxes identify chaetigers used for morphological/morphometric analyses (IV, XIII from prostomium and 10th from the pygidium).

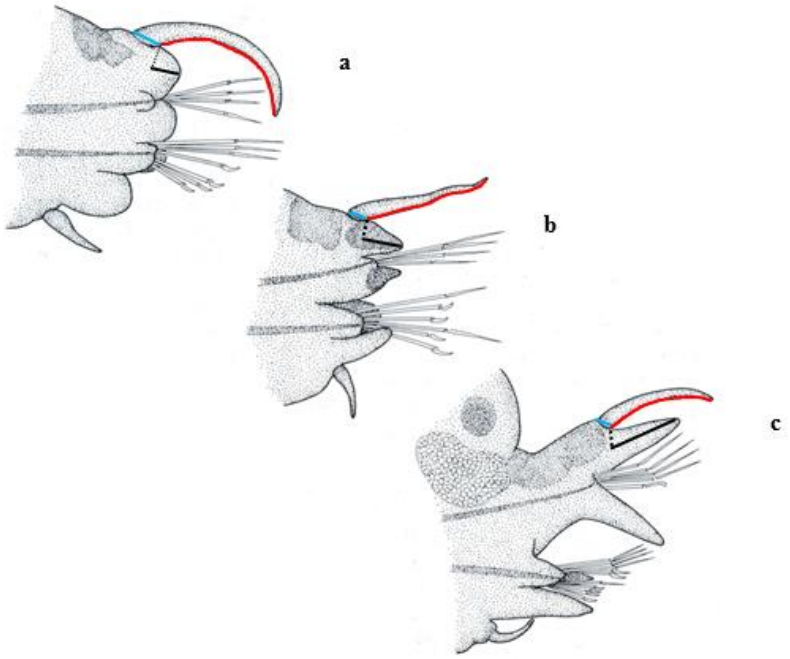

Figure 2.5 Graphic measurement scheme of parapodia at the level of the (a) anterior parapod, IV chaetiger from the prostomium; (b) intermediate parapod, XIII chaetiger from the prostomium; (c) posterior parapod, 10th chaetiger from the pygidium. Red lines identify the dorsal cirri lengths; light blue lines identify dorsal cirri widths; black line identify the lengths of the superior lobes (from Viéitez et al. 2004, modified).

2.3 Results

The biometric analysis performed on 30 specimens morphologically identified as *Platynereis dumerilii* (Kroeker et al. 2011) reveals that the body length (mm) is the best proxy to evaluate the size of the animals (biomass) (raw data R^2=0.9625) (Fig. 2.6a). In case of broken worms, the body width (mm) is an alternative parameter for the biomass evaluation (raw data R^2=0.8673) (Fig. 2.6b).

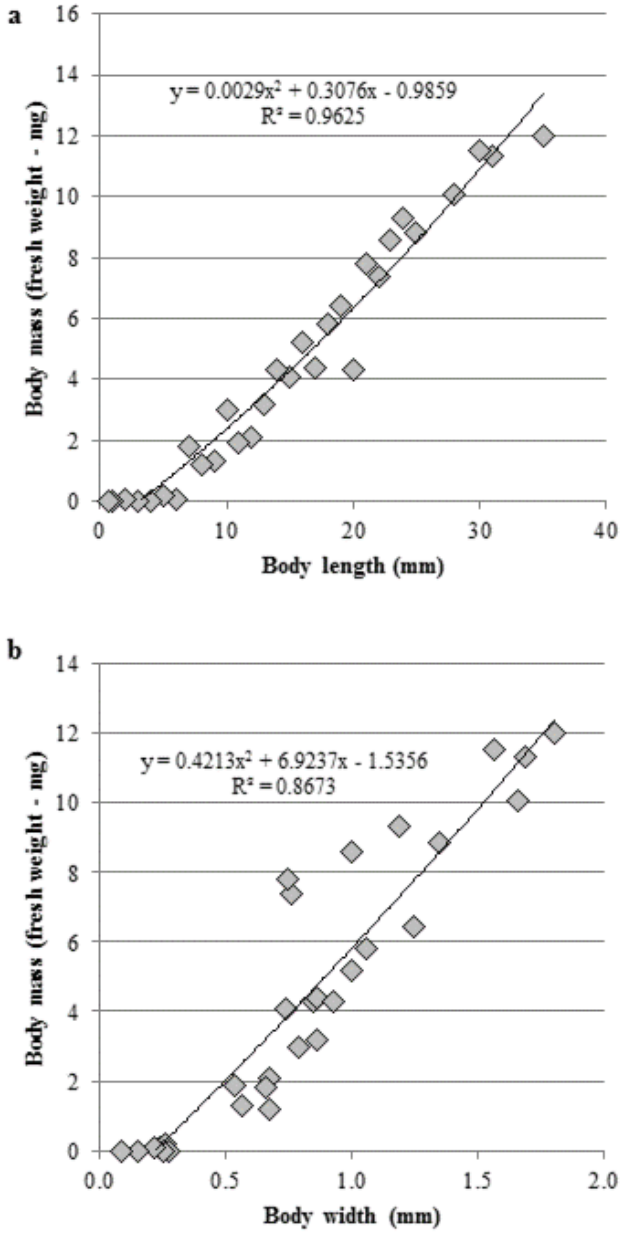

Figure 2.6 *Platynereis* spp. raw data of the biometric analysis: (a) body mass (w.w.) *vs* body length (mm); (b) body mass (w.w.) *vs* body width (mm).

The statistic analysis of the populations mean body masses (Fig. 2.7) reveals significant differences among populations, as reported in Tab. 2.1.

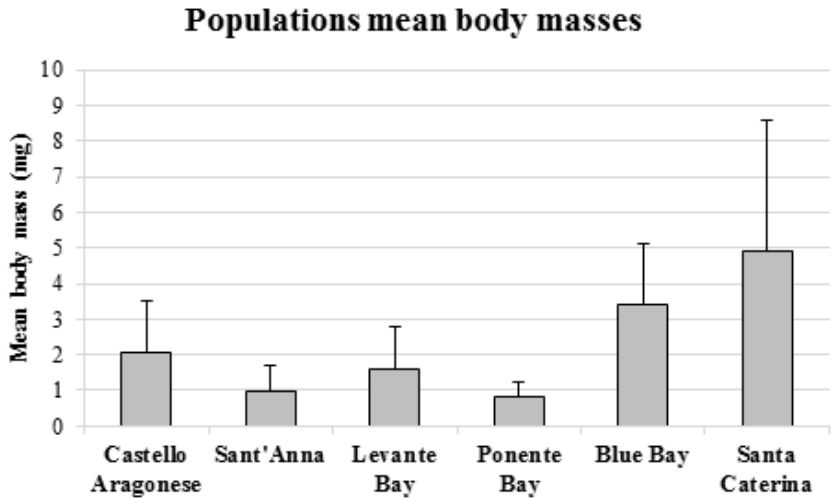

Figure 2.7 Populations mean body masses (w.w.) with standard deviation bars.

Table 2.1 *p*-values Dunn's multiple comparison test with Bonferroni method for the statistics analysis of populations mean body biomasses (***: $p < 0.001$; **: $0.001 < p < 0.01$; *: $0.01 < p < 0.05$; ns: $p \geq 0.05$).

	Castello Aragonese	Blue Bay	Levante Bay	Ponente Bay	Sant'Anna
Blue Bay	**				
Levante Bay	ns	***			
Ponente Bay	*	***	ns		
Sant'Anna	***	***	*	ns	
Santa Caterina	***	ns	***	***	***

The morphological analyses based on parapodia shape, homogomph falciger notochaetae shape and paragnaths arrangement, do not reveal any differences. Parapodia are characterized by the presence of a long dorsal cirrus and a shorter ventral one. The notopodial lobe shapes are rounded in the anterior chaetigers (IV body segment from the prostomium) and become more elongated and conical in the intermediate and posterior chaetigers (XIII body segments from the prostomium and 10th body segment from the pygidium) (Fig. 2.8). The homogomph falciger notochaetae are characterized by the same morphology in all the studied populations.

Both the anterior and posterior chaetae show elongated blade, sharply bent at the tip with a distal knob; below the tip, one side of the homogomph surface appears jagged (Fig. 2.9). The articulation is symmetrically at a right angle to the long axis of the shaft (Fig. 2.9). Regardless to the site of collection, the oral and maxillary pharynx rings are covered with paragnaths arranged in pectinate bars over both dorsal and ventral parts, but absent from areas I, II and V. Area III is characterized by several pairs of short bars; area IV has several short oblique and parallel bars; area VI has two short rows of lines; areas VII-VIII have up to five double bars arranged in two discontinuous rows.

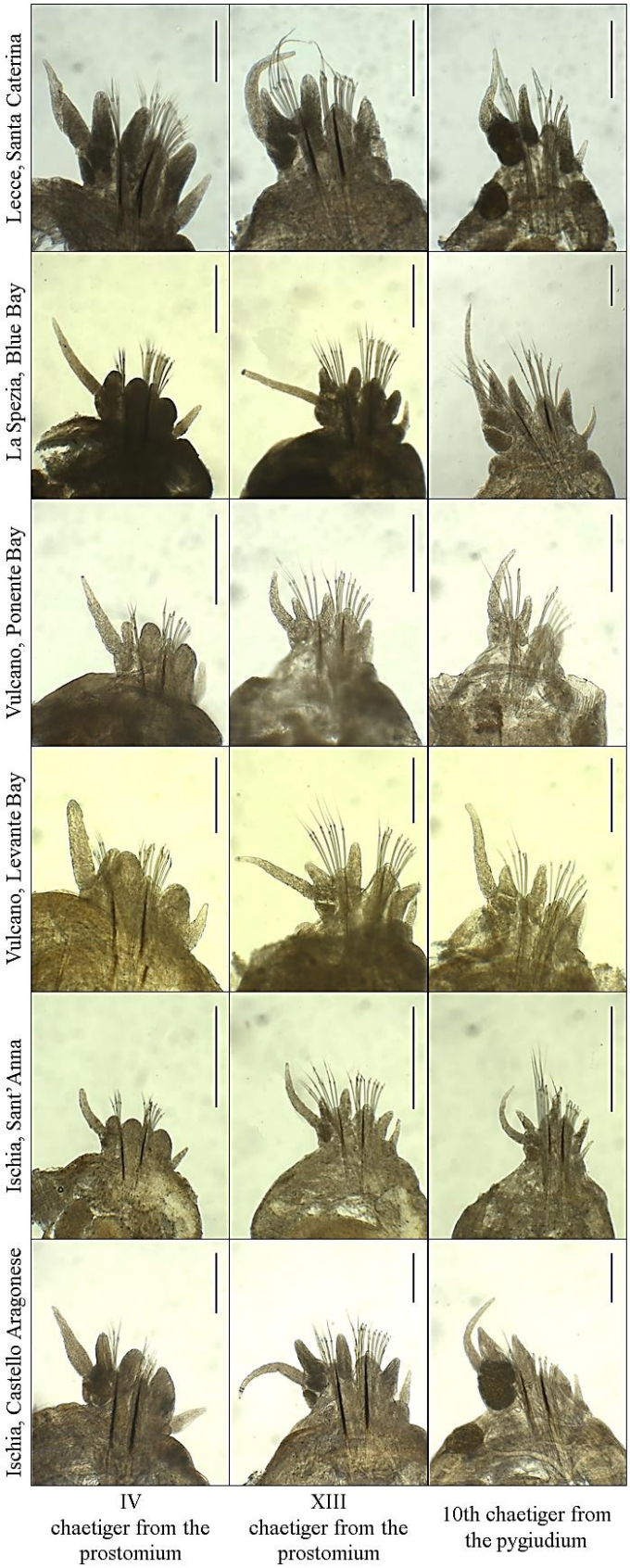

Figure 2.8 *Platynereis* spp. parapodia pictures (IV and XIII chaetiger from the prostomium and 10th chaetiger from the pygidium) from six different studied populations. Scale bars: 200 µm.

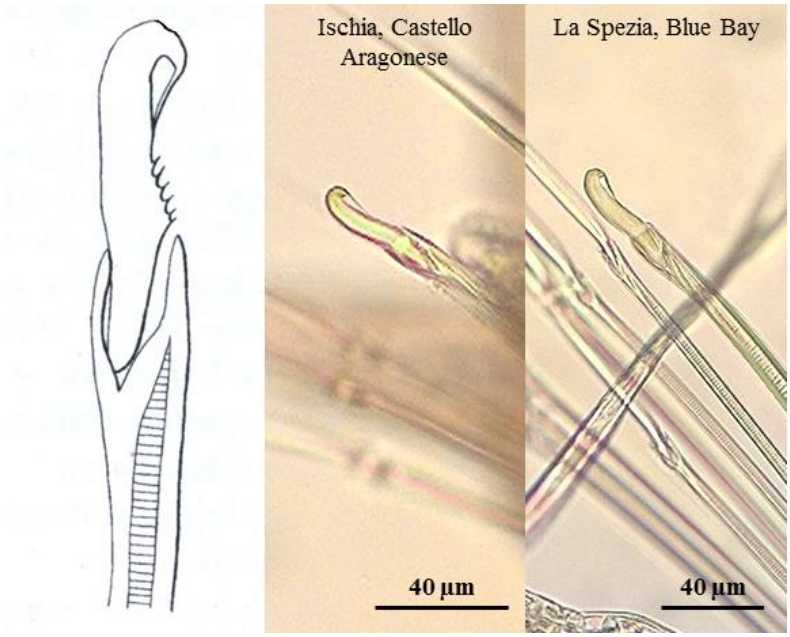

Figure 2.9 *Platynereis* spp. homogomph falciger notochaeta: shape scheme from Hartmann-Schröder 1996 (modified) and pictures from Castello Aragonese (Ischia Island) and Blue Bay (La Spezia) specimens.

The morphometric analysis performed on specimens belonging to *Platynereis massiliensis* (Castello Aragonese, south acidified areas) and *P. dumerilii* (Blue Bay) (see Results – Chapter 4) and comparable in body length, body width and number of chaetigers, reveals significant differences as reported in Tab. 2.2. Each measured element is from 20 to 30% smaller in putative *Platynereis massiliensis* individuals. The two species/populations are differentiated with little overlapping in the MDS analysis (Fig. 2.10). The ANOSIM non-parametric statistical test reveals a significant (p = 0.001) but low global R value of 0.203, indicating that the number of individuals analysed is sufficient and that the two species are quite similar. The SIMPER analysis highlights an intraspecific average similarity of 92.48% for *Platynereis massiliensis* and 91.05% for *P. dumerilii*. The average interspecific percentage of dissimilarity corresponds to 10.01 mainly due to the dorsal cirrus length of the XIII chaetiger from the prostomium (contribution of 19.09%), the 10th from the pygidium (contribution of 15.71%) and the IV from the prostomium (contribution of 14.08%) (Tab. 2.3).

Table 2.2 *p*-values PERMANOVA statistic analysis of *P. massiliensis vs P. dumerilii* morphometric parameters (***: $p < 0.001$; **: $0.001 < p < 0.01$; *: $0.01 < p < 0.05$; ns: $p \geq 0.05$).

	P. massiliensis vs P. dumerilii
Body length	ns
Body width	ns
n° of chaetigers	ns
IV from prostomium – dorsal cirrus length	***
IV from prostomium – dorsal cirrus width	**
IV from prostomium – superior lobe length	**
XIII from prostomium – dorsal cirrus length	***
XIII from prostomium – dorsal cirrus width	***
XIII from prostomium – superior lobe length	*
X from pygidium – dorsal cirrus length	**
X from pygidium – dorsal cirrus width	ns
X from pygidium – superior lobe length	ns
1st homogomph falciger notochaeta position	ns
1st homogomph falciger notochaeta length	***
Posterior homogomph falciger notochaeta length	***

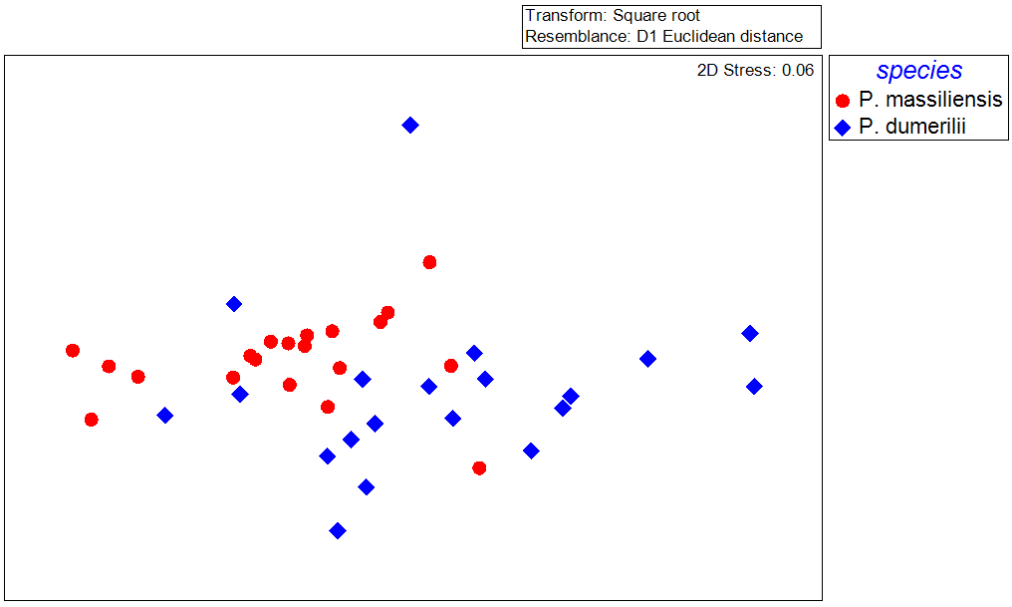

Figure 2.10 MDS analysis of *Platynereis* morphometric parameters from individuals genetically identified as *P. massiliensis* (Castello Aragonese, Ischia – red spots) and *P. dumerilii* (Blue Bay, La Spezia – blue spots).

Table 2.3 Grouped *Platynereis massiliensis* & *P. dumerilii* SIMPER dissimilarity analysis; average dissimilarity between groups = 10.01%.

	Group *P. massiliensis*	**Group *P. dumerilii***		
Species	Average. Abund.	Average Abund.	Average Dissimilarity	Contrib. %
XIII seg from prost Dorsal cirrus length	18.39	22.30	1.91	19.09
10th seg from pygidium Dorsal cirrus length	17.84	20.57	1.57	15.71
IV seg from prost Dorsal cirrus length	16.09	19.23	1.41	14.08
10th seg from pygidium Superior lobe length	11.46	11.51	1.08	10.80
XIII seg from prost Superior lobe length	11.68	13.24	0.95	9.45
IV seg from prost Superior lobe length	10.55	12.36	0.91	9.08
XIII seg from prost Dorsal cirrus width	7.06	8.23	0.55	5.47
IV seg from prost Dorsal cirrus width	7.08	7.96	0.52	5.18
10th seg from pygidium Dorsal cirrus width	6.67	7.22	0.50	5.03

2.4 Discussion

Ocean acidification, with elevated partial pressure of CO_2 in the seawater (also known as hypercapnia), is an important driver of change that can affect the marine biota resulting in a loss of biodiversity and ecosystems functions (Vizzini et al. 2017 and reference herein). Several studies have focused on how reduced pH may influence the metabolic rate (respiration) in marine invertebrates (Calosi et al. 2013b, Turner et al. 2015). It is considered the most fundamental of all biological rates since it controls the rates of energy uptake and allocation in different life-history traits such as survival, somatic growth and reproduction (Brown et al. 2004). A marked down regulation or 'metabolic depression' was registered on several marine ectotherms in low pH conditions (Michaelidis et al. 2005, Melatunan et al. 2011, Maas et al. 2012, Calosi et al. 2013b) and increased temperature (Daufresne et al. 2009) through the shutting down of expensive processes such as protein synthesis, which is translated by definition in reduced growth rates, adult body size and reproductive potential. To evaluate whether low pH may indirectly affect the body size of our sibling polychaete species, *Platynereis dumerilii* and *P. massiliensis*, six different *Platynereis* populations from naturally acidified and normal pH zones have been measured and compared in adult mean body sizes and biomasses.

Based on populations mean body biomasses, significant differences among populations were detected with the exception of: Levante Bay *vs* Ponente Bay, Levante Bay *vs* Castello Aragonese, Sant'Anna *vs* Ponente Bay, Santa Caterina *vs* La Spezia. On Ischia Island, specimens that thrived under acidified conditions (Castello Aragonese) showed significantly larger sizes ($p < 0.001$) than those from the control area (Sant'Anna). Our results contradict the previous observations made by Calosi et al. (2013b), in which the Castello Aragonese vent population showed a mean body mass (w.w.) much lower (80% lower) than specimens collected in the control area off Sant'Anna. The reduced body size was interpreted as a metabolic cost in conditions of chronic exposure to elevated pCO_2. The results of a recent paper (Garilli et al. 2015), in which the authors investigated the advantages of dwarfing in gastropods species living in naturally acidified conditions (vents off Vulcano Island) and normal seawater pH, support the conclusion made by Calosi et al. (2013b). Those defined as 'tolerant' species seems to be more efficient in the compensation of stressful environmental

conditions by increasing metabolic rates at the expense of body size (growth) (Garilli et al. 2015). Conversely, our results reveal that the Ischia vent specimens are slightly larger than those from the control site of Sant'Anna (Tab. 2.1). These differences are exclusively attributable to a pH effect, being the temperature of the two study sites the same. No significant differences were found between the acidified and control populations of Vulcano Island (both at ambient seawater temperature) suggesting the absence of pH effect on the body size and, therefore, a possible adaptation of the vent population to disturbed environment. The different latitude of sampling sites influences the seawater temperature affecting the main vital functions sich as development, growth and animal size and, in the specific case of ectotherms, the metabolic rates that scale according to the temperature (Gillooly et al. 2001). The increased metabolic rates associated with higher temperature are expected to reduce the ectotherms body size unless they can compensate somehow (greater food intake or reallocation of energies) (Bickford et al. 2010). Organisms must split their energy stock between the main vital functions, such as physiological maintenance, growth and reproduction, and the first thing that can be limited is growth in favour of other functions. The larger mean body mass reported for the Blue Bay (La Spezia) population, might be explained as a consequence of the lower temperature range typical of the Ligurian Sea, in the northern part of the western Mediterranean. The relatively cold water regime of La Spezia allows polychaetes to invest their energies in body growth. An overall mean body size reduction can be observed in the insular populations (Ischia and Vulcano) when compared with the ones from the mainland (Santa Caterina and La Spezia), regardless of pH conditions (Fig. 2.4). This might be explained as a sort of dwarfisms effect correlated with the island systems. The restricted habitat of the islands could induce an energetic limitation due to low absolute food availability ('resource limitation mechanisms' by Lomolino 1985), causing a fauna miniaturization as result of resource-constrained environments.

Morphological analyses (parapodia shape, homogomph falciger notochaetae shape and paragnaths arrangement) have been carried out in order to evaluate whether different pH conditions, geographical distribution and eventually reproductive isolation can be translated into morphological differences between separated populations, but no dissimilarities have been detected. The parapodia shape and paragnaths arrangement correspond with the morphological description made by Viétez et al. (2004). The

homogomph falciger notochaetae shape is the same as described by Day (1967) with a jagged lateral surface, as reported also by Viétez et al. (2004) and Hartmann-Schröder (1996) (Fig. 2.6).

Based on the population genetic results (see Chapter 4), it has not been possible to consider the geographical origin as a distinctive feature to discern species; our genetic results demonstrate that we analysed actually at least four different species, grouped in two complexes of siblings, that often sympatrically coexist in the same areas. The COI barcoding approach clearly highlights that most of the Castello Aragonese specimens and all of those collected in Blue Bay (La Spezia) putatively belong to *Platynereis massiliensis* and *P. dumerilii*, respectively. The morphometry on these two species/populations reveals the occurrence of few significant dissimilarities in the dorsal cirrus length of the XIII chaetiger from the prostomium, the 10th from the pygidium and the IV from the prostomium (Tab. 2.3). We recall that we compared individuals belonging to the same size range (Tab. 2.2), therefore *Platynereis dumerilii* individuals (Blue Bay population) are characterized by larger sizes of the morphometric parameter considered. This analysis suggests the occurrence of few morphometric (morphological) differences between *Platynereis dumerilii* and *P. massiliensis*.

Chapter 3

Reproductive biology and gamete morphology analysis of *Platynereis* spp. populations in relation to Ocean Acidification

3.1 Introduction

Ocean acidification is considered one of the major threats to the marine ecosystems (Fabry et al 2008). Anthropogenic atmospheric carbon dioxide (CO_2) emissions are leading to a modification in the seawater carbonate chemistry with a resulting pH reduction (Gattuso and Buddemeier 2000, Caldeira and Wickett 2003, Feely et al. 2004). Most scientific research to date has focused on the effects of OA on calcifying organisms, since presumably they will be the most affected group among marine organisms by the consequences of high pCO_2 in seawater (Orr et al. 2005, Fabry et al. 2008). However, changes in non-calcareous taxa may also occur (Connell and Russell 2010, Kroeker et al. 2010) and the consequences of this phenomenon can be directly investigated in submarine volcanic CO_2 vent systems that represent a great resource to evaluate the effects of exposure to low pH conditions on the marine biota (Hall-Spencer et al. 2008).

Polychaetes are one of the most abundant taxonomic groups of the first CO_2 vents marine benthic community investigated, at Castello Aragonese off Ischia (Italy) (Kroeker et al. 2011). This class of organisms is characterized by reproductive and developmental modes that are conspicuously variable even among morphologically similar congeneric species. Many examples of intraspecific variability of the reproductive biology strategies have highlighted the existence of sibling species complexes, the members of which have species-specific modes of reproduction and development (Grassle and Grassle 1976, Rice and Simon 1980, Wilson 1983, Pfannenstiel et al. 1987, Manchenko and Radashevsky 1993, 2002, Sato 1999, Kruse and Reise 2003, Kruse et al. 2003, Sato and Nakashima 2003, Kikuchi and Yasuda 2006, Lewis and Karageorgopoulos 2008, Paxton and Åkesson 2010). These important findings have been made possible by comparative studies of reproduction and development of species that were initially identified as a single entity. Lucey et al. (2015) recently published a study that confirms the occurrence of different reproductive biology strategies between sibling species. The authors hypothesized that invertebrate species, mainly polychaete, with a brooding reproductive strategy take advantage under OA conditions as do those occurring in CO_2 vent areas (Lucey et al. 2015). Reproductive biology observations in laboratory conditions and experiments of cross-fertilization among specimens identified as *Platynereis dumerilii* (Audouin and Milne-

Edwards, 1834) collected within and outside the vents of Ischia Island, demonstrated the presence of two different sibling species with different reproductive habits. Free spawning reproduction with epitokous transformation (typical of *P. dumerilii*) was observed in specimens from the normal pH zone, while eggs brooding inside the tube and protrandric hermaphroditism occurred in specimens thriving in the naturally acidified area of the Castello vent system (Lucey et al. 2015) (Tab. 3.1). Laboratory rearing of a male individual at a reproductive mature and pelagic adult stage, collected from the control area (normal pH), together with a sexually immature adult specimen of *Platynereis* spp. from the acidified site, resulted in deposition of eggs inside the tube by the vent individual (likely due to a pheromone-induced response between the two sexes). This cross-breeding was translated into fertilization, but not into developed eggs (Lucey et al. 2015). These results, coupled with the outcome of a COI barcoding approach, revealed that the vent population belonged to the neglected sibling species of *Platynereis dumerilii*, *P. massiliensis* (Moquin-Tandon 1869) (Lucey et al. 2015, Valvassori et al. 2015). The two sibling species are indistinguishable at the sexually immature adult stage but they differ for the reproductive biology, gamete morphology and embryogenesis (see Table 1) (Hauenschild 1951, Schneider et al. 1992). *Platynereis dumerilii* is a gonochorostic species with a semelparous reproduction, characterized by a sexual metamorphosis that invokes a transition from an immature benthic atokous condition to a drastically changed pelagic and sexually mature epitokous form, called heteronereis. The single spawning event, synchronized with the full moon, implies the gamete release in the water column and the successive death of both male and female individuals (Fischer and Dorresteijn 2004). Conversely, *Platynereis massiliensis* (formerly called nereidogenic form of *Nereis dumerilii* by Hauenschild 1951) is the closest relative of *P. dumerilii*. It is a protandric sequential hermaphrodite species with an iteroparous reproduction. The female lay eggs inside the tube where fertilization occurs; later the female dies and the embryos are oxygenated and protected by the ventilation movements of the male. The embryos undergo semi-direct development into young worms (Schneider et al. 1992). One of the main distinctive features between the two siblings is the shape and size of sexual products (Hauenschild 1951, Schneider et al. 1992). Mature adult individuals of *Platynereis dumerilii* are characterized by coelom full of oocytes of less than 180 μm in diameter or spermatozoa with spherical and shorter heads (Lücht and Pfannenstiel 1989).

On the other hand, the coelom of mature adults belonging to *Platynereis massiliensis* contains yellow-orange oocytes of more than 250 μm in diameter and/or spermatozoa with long and cylindrical heads (Hauenschild 1951, Lücht and Pfannenstiel 1989). The volume of *Platynereis massiliensis* eggs is greater than *P. dumerilii* ones due to the higher yolk content including protein yolk bodies and lipid droplets (86-93% of the egg volume in *P. massiliensis*, 64% in *P. dumerilii*) (Schneider et al. 1992).

Table 3.1 Overview of the main differences in life history traits among *Platynereis dumerilii* and *P. massiliensis*.

Platynereis dumerilii	***Platynereis massiliensis***
Gonochoric	Protandric hermaphrodite
Semelparous	Iteroparous
Epitoke (heteronereis stage)	No epitoke
Free spawner	Brooder
Oocyte size < 180 μm	Oocyte size > 250 μm
Spherical and shorter sperm head	Long and cylindrical sperm head

Lucey et al. (2015) clearly showed the co-occurrence of the two *Platyneris* siblings species in Ischia Island but with different distribution patterns interpreted as the advantage of a life-history strategy over the other. The brooding *Platynereis massiliensis* prevailed inside low pH habitats while the broadcasting *P. dumerilii* was more abundant in normal pH conditions. The parental care strategy of the neglected sibling was therefore recognised as the winning life-strategy in stressful conditions of OA (Lucey et al. 2015).

Based on the above mentioned differences in life history traits between the two *Platynereis* sibling species, the intended aim of this work is to study the reproductive biology and the gamete morphology of seven different populations of *Platynereis* spp. collected from both acidified and normal pH areas situated along the Italian coasts. Considering a larger spatial-scale which takes into account at least three CO_2 naturally vent systems, we want to verify whether effectively parental-care life history strategies are favored and selected against low pH conditions. The purpose of this chapter is also to verify, through the conduction of cross-breeding experiments, whether specimens characterized by the same reproductive strategy and collected in geographically separated naturally acidified sites, are reproductively connected or isolated.

3.2 Materials and methods

3.2.1 Detailed life history traits and reproductive biology strategies of the target species: Platynereis dumerilii *and* P. massiliensis

Platynereis dumerilii is a model polychaete species that has been bred in the laboratory since 1953 without interruption (Fischer et al. 2010). It is considered a cosmopolitan species inhabiting shallow hard bottoms and seagrass meadows; in the Mediterranean Sea it reproduces from late spring to early summer (Giangrande et al. 1989, 2002). Eggs, embryos and larvae are transparent and eggs measure approximately 160 μm each in diameter (less than 180 μm). Its larval development can be subdivided, as traditionally recognized for polychaetes, into three major stages: trochophore, metatrochophore and nectochaetae (Fischer et al. 2010) (Fig. 3.1). An additional staging system, currently used to describe *Platynereis dumerilii* developmental steps, is related to the hours post fertilization (hpf) at a constant temperature of 18 °C (Dorresteijn et al. 1993). One of the most recent and complete morphologically based studies correlated with the above mentioned hpf for this model organism was carried out Fischer et al. (2010) (see Table 3.2 as an overview of the metamorphoses of *Platynereis dumerilii*). Between 0 and 24 hpf we can talk about 'embryonic development', which refers to the transition from the fertilized egg to the prototrochophore. The term 'prototrochophore' (introduced by Häker 1897) is used to describe a pre-larva, as an earlier stage of the trochophore, characterized by a preoral band of short cilia without mouth and anus. The trochophore stage (24-48 hpf) is a pelagic and non-feeding larval stage with spherical shape characterized by the occurrence of an equatorial ciliary belt, called prototroch, and larval eyes. Thanks to the beating cilia, this larval stage is able to swim in the water column, rotating around its anteror-posterior axis (Fischer et al. 2010). The metatrochophore (48-66 hpf) is a conical three-segmented and non-feeding larva that maintains the pelagic and helical swimming with positive phototaxis of the previous stage, while rapidly developing bilateral and segmental structures occur (e.g. chaetae, ciliary bands, etc.) (Fischer et al. 2010). The nectochaetae larva (66 hpf to 7 days old) is a non-feeding 3-body segment larval stage fully developed with parapodia and chaetae.

For the first time the worms are able to swim in a straight direction without rotation and show a mixed pelago-benthic lifestyle (Fischer et al. 2010). The nectochaetae is followed by errant juvenile self-sustained stages with 3-5 body segments, able to move around with undulating movements. The pelagic larval stages last for 1 week according to Fischer et al. (2010). These stages are characterized by the completion of the settlement metamorphosis, the beginning of a cephalic metamorphosis and, thanks to the spinning glands, the worms start to build their own tube (Fischer et al. 2010) (Fig. 3.1). The small atokous worms (from completion of cephalic metamorphosis to fully-grown adult), with increased growth rates, leave their tubes occasionally in case of stress or whether they need food (Fischer et al. 2010). The following large atokous worms are characterized by more than 50 chaetigers; they start to produce gametes in the coelom that will became mature once the animals have approximately 70 body segments and the growth rate becomes slower. In laboratory conditions, the lifespan from fertilization to maturity of *Platynereis dumerilii* specimens goes from 3 to about 18 months (on average of 6/7 months) at 18 °C (Fischer et al. 2010). Finally, the adult individuals undergo a process known as 'sexual metamorphosis', which represents the transition from an immature benthic atokous condition to a pelagic sexually mature epitokous one, called heteronereis stage (Fig. 3.1). This transformation implicates a series of drastic morphological and physiological changes such as the feeding interruption with gut collapse, an increase in eye size, trunk subdivision in two parts with different shapes of parapodia (to favour fast swimming) and change in body colour according to the animal gender (Fig. 3.2). The females become yellow due to the yellow colour of the mature oocytes (Fig. 3.2). On the contrary, male specimens become white in the anterior half of the body due to the occurrence of spermatozoa masses, and red in the posterior half as a result of a large number of bloody capillaries (Fischer et al. 2010) (Fig. 3.2). The sexually mature heteronereis females and males (females are mature only for a few hours, males for slightly longer than one day), synchronized by lunar periodicity (Zantke et al. 2013) and attracted to each other by pheromones (Zeeck et al. 1988, 1998), become pelagic and start to swim rapidly, this behaviour is known as 'swarming'. The swarming ends with the nuptial dance in which males and females, swimming in circles, deliver respectively spermatozoa (from posterior papillae) and eggs (from fissures among chaetigers) in the water column allowing the fertilization (Fischer et al. 2010). After the spawning event, both males and females die.

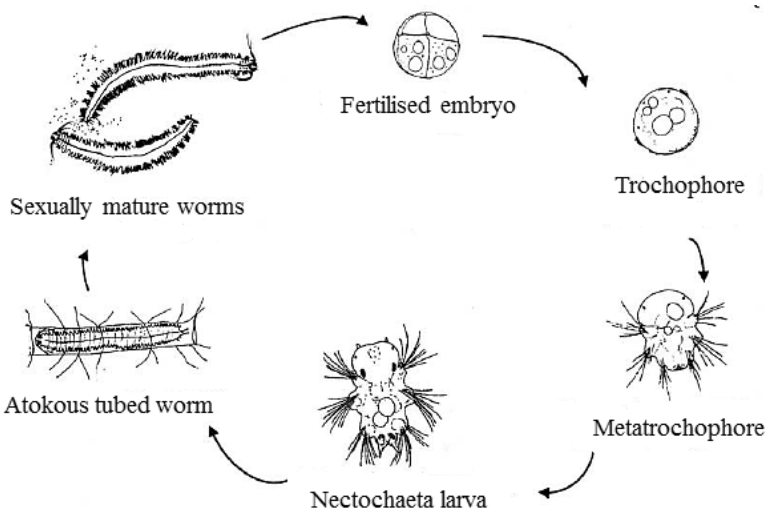

Figure 3.1 *Platynereis dumerilii* life cycle scheme: fertilized embryo; trochophore, metatrochophore and nectochaetae larval stages; adult atokous worm inside the tube; epitokous sexually mature worms.

Figure 3.2 Habitat and spawning of *Platynereis dumerilii*. Below: an immature worm (‘atoke’). Middle left: a mature (‘epitokous’) male swimming in search of a female. Top and upper right: encounter of mature worms (upper right: male; top: female) starting their rapid ‘nuptial dance’ (from Fischer and Dorresteijn 2004).

Table 3.2 Overview of the metamorphoses of *Platynereis dumerilii* showing the lifestyle before and after metamorphosis, as well the corresponding morphological changes (from Fischer et al. 2010).

	Lifestyle before metamorphosis	Lifestyle after metamorphosis	Morphological changes
Settlement metamorphosis	planktonic	errant	Digestive tract becomes functional, ciliary bands start to get abolished, apical tuft is lost.
Cephalic metamorphosis	errant	tubicolous	Transformation of the first pair of parapodia, formation of tubes, larval eyes and ciliary bands disappear.
Sexual metamorphosis	tubicolous	pelagic	Maturation of gametes, enlarging adult eyes, development of paddle shaped chaetae and epitokous musculature.

Platynereis massiliensis, recognized as the closest relative of *P. dumerilii*, is a protandric hermaphrodite species with a semi-direct development and without free swimming stages (Hauenschild 1951). The developmental process of this species has been recently described by Helm et al. (2014), establishing a staging system based on the morphological juvenile features. The early developmental stage, identified as stage 0, is characterized by a large amount of yolk and a spherical shape (Fig. 3.3 A). At stages 1 and 2 two pairs of eyespots appear, the body is segmented in 3 chaetigers and both anterior and posterior ends are formed (Fig. 3.3 B). At stage 3, the anterior and posterior regions differ in prostomial and pygidial regions separated by a yolky ovoid trunk (Fig. 3.3 C). At stage 4 the juvenile body is composed of 3-5 chaetigers, anterior and anal cirri and dorsal blood vessels (Fig. 3.3 D). Prominent dorsal and ventral anterior cirri, developing antennae and 5-8 body segments, characterize stage 5 (Fig. 3.3 E). At stage 6, the body consists of 8-11 segments with yolk remnants, antennae and palps are completely formed (Fig. 3.3 F-G). In the last stage (stage 7, Fig. 3.3 H-I) specimens fully acquire the typical adult body shape without yolk residuals; after about 4 weeks of lecithotrophic development they start feeding, leave the parental brood tube and build their own (Schneider et al. 1992, Helm et al. 2014). Sexual development starts early in life with maturing females that reinforce their own tube giving it a brownish-yellow appearance. As soon as females approach the egg-laying, becoming less active, male worms go inside the tube side by side with the females. The eggs are laid in a monolayer on the side of a second thin tube, which is inside the brood tube.

After the sexual interaction (around dusk) the female leaves the tube and within one day dies (Schneider et al. 1992). The undulating male guarantees a proper oxygenation and protection to the developing eggs/juveniles (Schneider et al. 1992, Lucey et al. 2015).

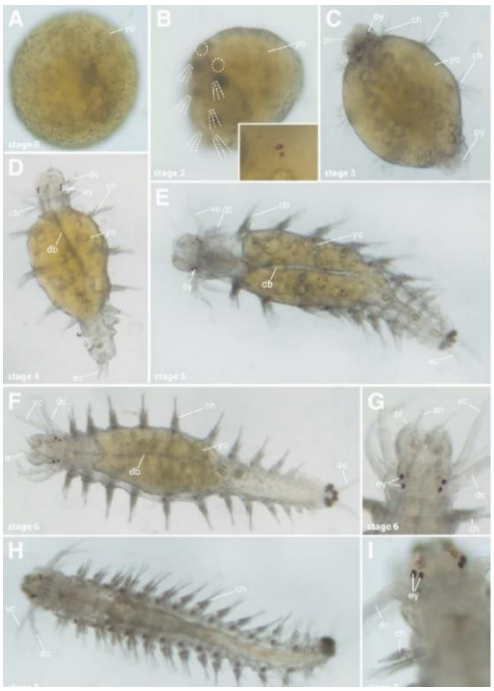

Figure 3.3 General development of *Platynereis massiliensis* illustrated by light microscopic images: (A) stage 0; (B) stage 2; (C) stage 3; (D) stage 4; (E) stage 5; (F-G) stage 6; (H-I) stage 7 (from Helm et al. 2014).

3.2.2 Sample collection and processing

Specimens of *Platynereis* spp. were sampled in association with macroalgae, collected by snorkelling and/or SCUBA diving in 2015 and 2016. A standardized collection was performed in each of the study area: Blue Bay (La Spezia, normal pH), Castello Aragonese (Ischia, low pH), Sant'Anna (Ischia, normal pH), Levante Bay (Vulcano, low pH), Ponente Bay (Vulcano, normal pH), Ditella Hot/Cold points (Panarea, low pH), Santa Caterina (Lecce, normal pH). Within one hour after collection, macroalgae were transported to the laboratory and *Platynereis* spp. specimens, identified thanks to the typical sinuous swimming movement, were collected and inserted in 100 ml evaporating dishes filled with filtered (0.22 µm) seawater (up to 5 specimens in each bowl).

In order to avoid an excessive evaporation and increased salinity, the culture dishes were placed over lab bench inside plastic containers with blotting paper soaked with distilled water in the bottom, and closed with a lid without aeration. The cultures were kept inside a thermostatic chamber and were reared in a summer regime of temperature (21±1 °C), light and long day photoperiod (L:D = 16h:8h) to stimulate the breeding. Once a week the cultures were inspected to check the reproductive status, to change water and supply food (fragmented deep-frozen spinach). As a whole, 65 specimens from Blue Bay, 172 specimens from Ischia Island (122 from Castello Aragonese and 50 from Sant'Anna), 112 specimens from Vulcano Island (68 from Levante Bay and 44 from Ponente Bay), 15 specimens from Panarea Island and 96 specimens from Santa Caterina, were reared in the lab. Reproduction and developmental stages were followed and documented with the stereomicroscope Leica MZ125 and the optical-microscope Leitz Dialux 20 EB with a micrometric ocular (2 mm). For each brooding specimen, reproductive output and timing, eggs size and juvenile development were recorded taking photographs and measuring them.

The mature spermatozoa morphology and ultrastructure were investigated by both transmission electron microscope (TEM) and scanning electron microscope (SEM). For TEM observations, two sexually mature specimens of *Platynereis dumerilii* (from Sant'Anna and Santa Caterina) and two of *P. massiliensis* (from the Castello Aragonese vents) were primarily fixed in 2.5% glutaraldehyde. For SEM analyses, spermatozoa were collected by the posterior end of the body of three *Platynereis massiliensis* individuals (from the Castello Aragonese vents) and fixed in 1% glutaraldehyde. The sample preparation protocols for both analyses are summarized in Tables 3.3 and 3.4.

Table 3.3 *Platynereis* spp. standard fixation and embedding protocol for resin section TEM.

Primary fixation	2.5% glutaraldehyde diluted with filtered (0.22 µm) seawater	Overnight at +4°C
Buffer Rinse	Filtered seawater rinse	3 times 10 minutes each
PostFixation	1% osmium tetroxide diluted with distilled water	1 hour at RT
Buffer Rinse	Distilled water rinse	3 times 10 minutes each
Dehydration	30% ethanol	30 minutes
	50% ethanol	30 minutes
	70% ethanol	30 minutes
	90% ethanol	30 minutes
	100% ethanol	3 times 30 minutes each
Resin embedding	Propylene oxide	20 minutes
	Resin infiltration 1:1 mix of propylene oxide:resin	Overnight
	Resin embedding (Epon 812)	Overnight at RT
	Polymerise in stove at 60°C	48 hours
Slices preparation	Leica Ultracut UCT ultramicrotome to cut 70 nm slices	

Table 3.4 *Platynereis* spp. standard fixation protocol for SEM.

Primary fixation	1% glutaraldehyde diluted with filtered (0.22 µm) seawater	Overnight at +4°C
Buffer Rinse	Filtered seawater rinse	3 times 10 minutes each
PostFixation	1% osmium tetroxide diluted with distilled water	1 hour at RT
Buffer Rinse	Distilled water rinse	3 times 10 minutes each
Dehydration	30% ethanol	30 minutes
	50% ethanol	30 minutes
	70% ethanol	30 minutes
	90% ethanol	30 minutes
	100% ethanol	3 times 30 minutes each
Critical Point Drying	Bring samples to the critical point dryer in 100% ethanol	2 hours
Sputtering	Heavy metal sputtering	

Finally, three different cross-breeding tests were performed, coupling specimens from the acidified sampling sites, respectively: Castello Aragonese *vs* Levante Bay; Castello Aragonese *vs* Panarea; Panarea *vs* Levante Bay.

3.3 Results

Reproductive biology observations highlight the occurrence of two different reproductive biology patterns (see Tab. 3.5). Specimens collected from Blue Bay (n = 21), Sant'Anna (n = 18) and Santa Caterina (n = 13), underwent a drastic morphological change in an epitokous/heteronereis free spawner form with enlarged eyes, paddle-like chaetae and changed parapodia. The trunk was splitted into two parts and the body colour of the animals changed according to the gender: females appeared yellow and males exhibited white and red colouring. The same sexual metamorphosis was also observed for a few individuals collected in both Ponente Bay (n = 2) and Panarea (n = 2). On the other hand, few specimens from Santa Caterina, previously fixed in 4% formalin for the morphological and morphometric analyses (see Chapter 2), showed the adult atokous morphological shape with mature spermatozoa or large yolky oocytes. Specimens coming from the acidified areas of Castello Aragonese and Levante Bay showed a brooding behaviour laying eggs inside their tube. Sixteen *Platynereis* individuals collected in Castello Aragonese were observed to brood. The eggs were spawned by the females inside an inner small microtube, located inside the parental one (Fig. 3.4a). The eggs size ranged from 250 to 350 μm in diameter (sample size = 21, sample mean = 308.95 μm; σ = 27.38) according to the embryo developmental stage. They appeared full of yolk (Fig. 3.4b) and were oxygenated by the ventilation movement of the males inside the brood tubes. After laying eggs, females were observed going away from the parental tube and diying after few hours. The large yolky eggs hatched approximately a week and a half/two weeks after being laid in spherical early stages, followed by juveniles with 3 body segments each (Fig. 3.4c). Juveniles with 5-6 chaetigers still had remaining body yolk and they occasionally left the parental tube (Fig. 3.4d). Once reaching approximately 10 body segments (about 1 month old) they started feeding autonomously and built their own tube. Sixteen *Platynereis* specimens collected in Levante Bay showed the same brooding behaviour (Fig. 3.5a-f). The parents ventilated the eggs which ranged in size between 250-350 μm (sample size = 33, sample mean = 292.21; σ = 26.63) (Fig. 3.5c) and hatched with spherical and then 3-segment juveniles (Fig. 3.5d), as observed for the specimens from Ischia. The same brooding behaviour, eggs size and semi-direct development were also observed for specimens from Panarea (n = 9; Fig. 3.6a-c) and Ponente Bay (n = 10).

Table 3.5 Summary of the observed reproductive behaviours under laboratory controlled conditions.

Sampling site	Reproductive behaviour
La Spezia, Blue Bay	Free spawners
Ischia Island, Castello Aragonese	Brooders
Ischia Island, Sant'Anna	Free spawners
Vulcano Island, Levante Bay	Brooders
Vulcano Island, Ponente Bay	Brooders + Free spawners
Panarea Island, Ditella	Brooders + Free spawners
Lecce, Santa Caterina	Brooders + Free spawners

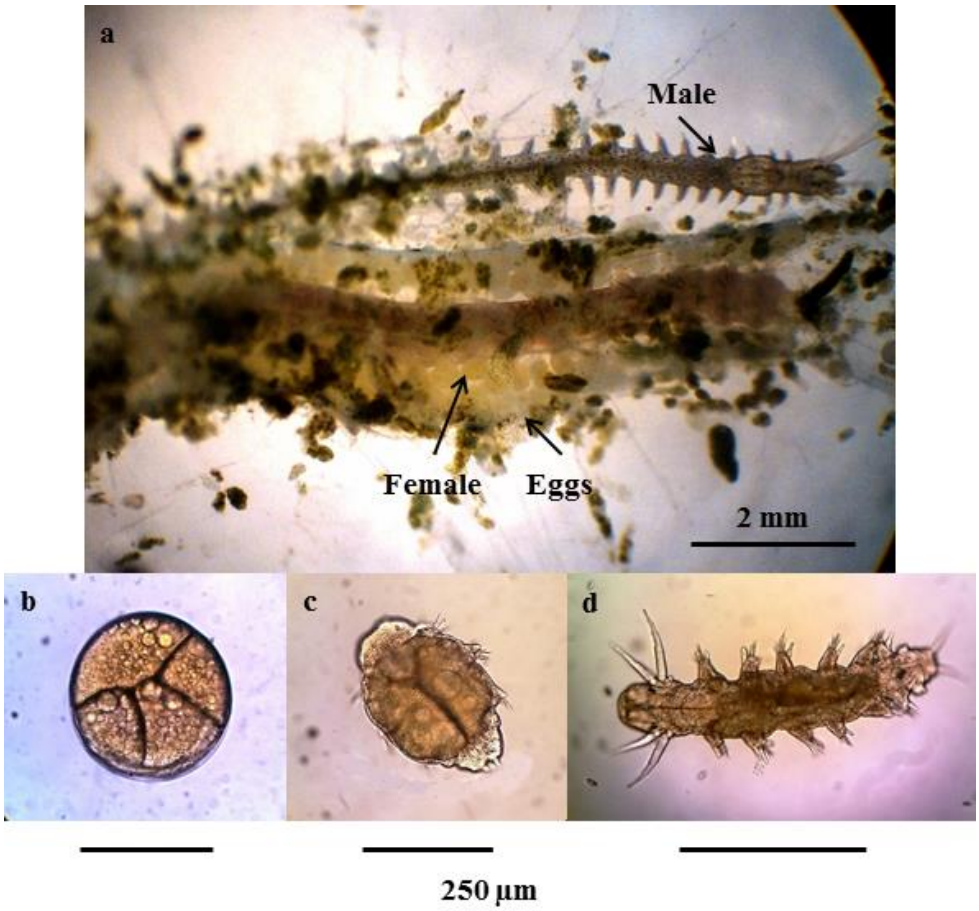

Figure 3.4 *Platynereis massiliensis*-like from the Castello Aragonese S3/S2 vent areas (Ischia Island) with brooding behaviour: (a) female specimens inside the brooding tube with the laid eggs); (b) the developing embryo inside the egg; (c) 3-segment juvenile rich in yolk; (d) 6-segment juvenile with some yolk remnants (from Wäge et al. 2017).

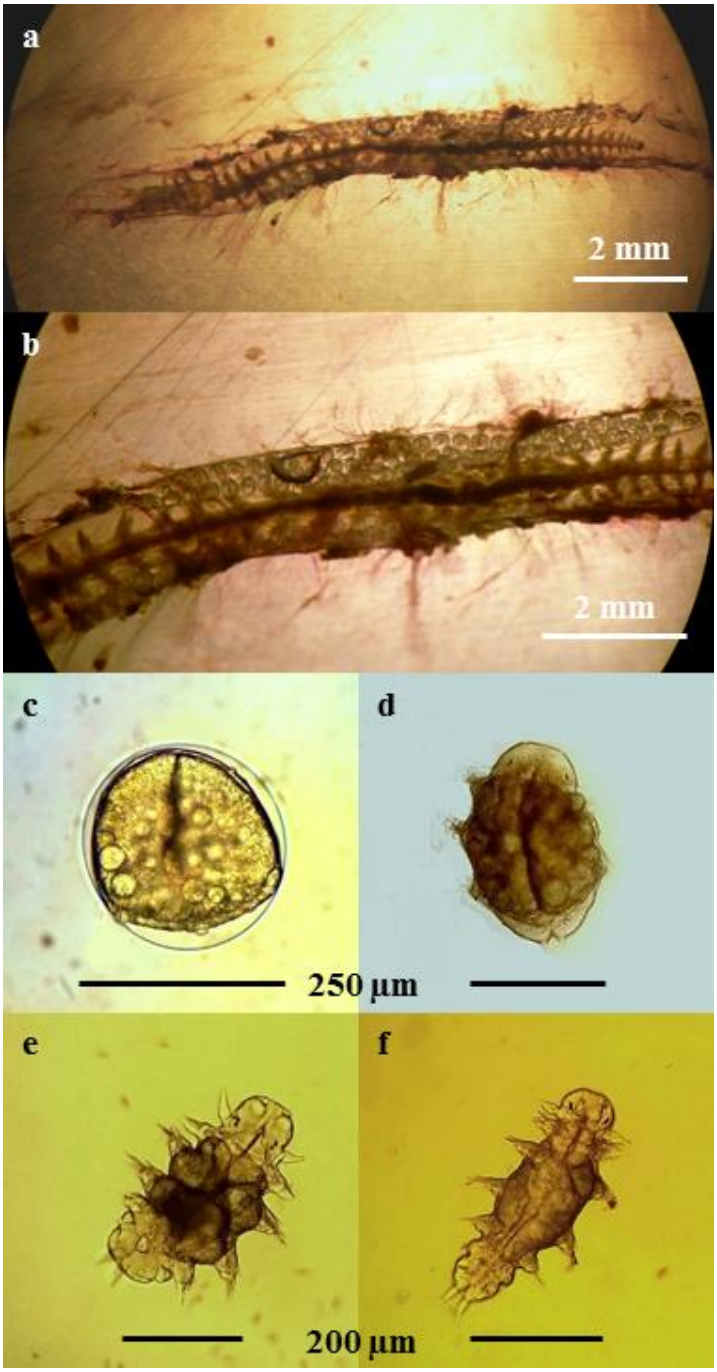

Figure 3.5 *Platynereis massiliensis*-like from the S3 vent site off the Levante Bay (Vulcano Island): (a-b) parent specimen inside the brooding tube with laid eggs; (c) a laid egg; (d) 3-segmented juvenile; (e) 4-segmented juvenile; (f) 5-segmented juvenile (from Wäge et al. 2017).

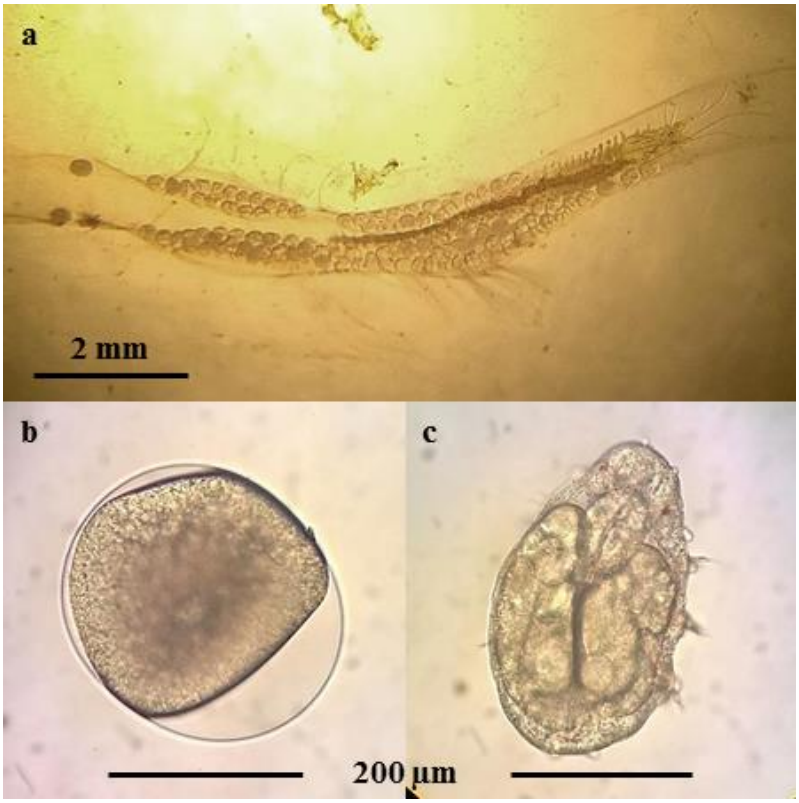

Figure 3.6 *Platynereis massiliensis*-like from the vent site off the Ditella beach Hot/Cold points (Panarea Island): (a) parent specimen inside the brooding tube with laid eggs; (b) a laid egg; (c) 3-segment juvenile.

In the cross-breeding tests (see Methods above), only one egg deposition was observed with specimens from Castello Aragonese and Levante Bay, but the fertilized eggs immediately stop developing.

TEM and SEM observations have been made on brooding specimens from Castello Aragonese (putative *Platynereis massiliensis*) and broadcasting individuals from Sant'Anna and Santa Caterina. Male gametes of the brooding species are characterized by an elongated head of about 5.5 µm (mean value; n = 23, σ = 0.46) in length, 0.9 µm (mean value; n = 23, σ = 0.08) in diameter and rounded acrosome of approximately 13% of the head length (Fig. 3.7 and 3.8). The flagellar membrane is characterized by two lateral projections (folds, as described in Lücht and Pfannenstiel 1989) that disappear in the distal part of the flagellum (Fig. 3.7c and 3.8b-c). The microtubules of the flagellum show the typical 9+2 arrangement.

Conversely, broadcasting specimens (putative *Platynereis dumerilii*) spermatozoa have a spherical head of about 2.6 μm in length (mean value; n = 10, σ = 0.32) and 1.8 μm in diameter (mean value; n = 10, σ = 0.13) and the acrosome of 25% of the head length (approx.). The lateral folds of the flagellum and the microtubule 9+2 arrangement are present in these spermatozoa too.

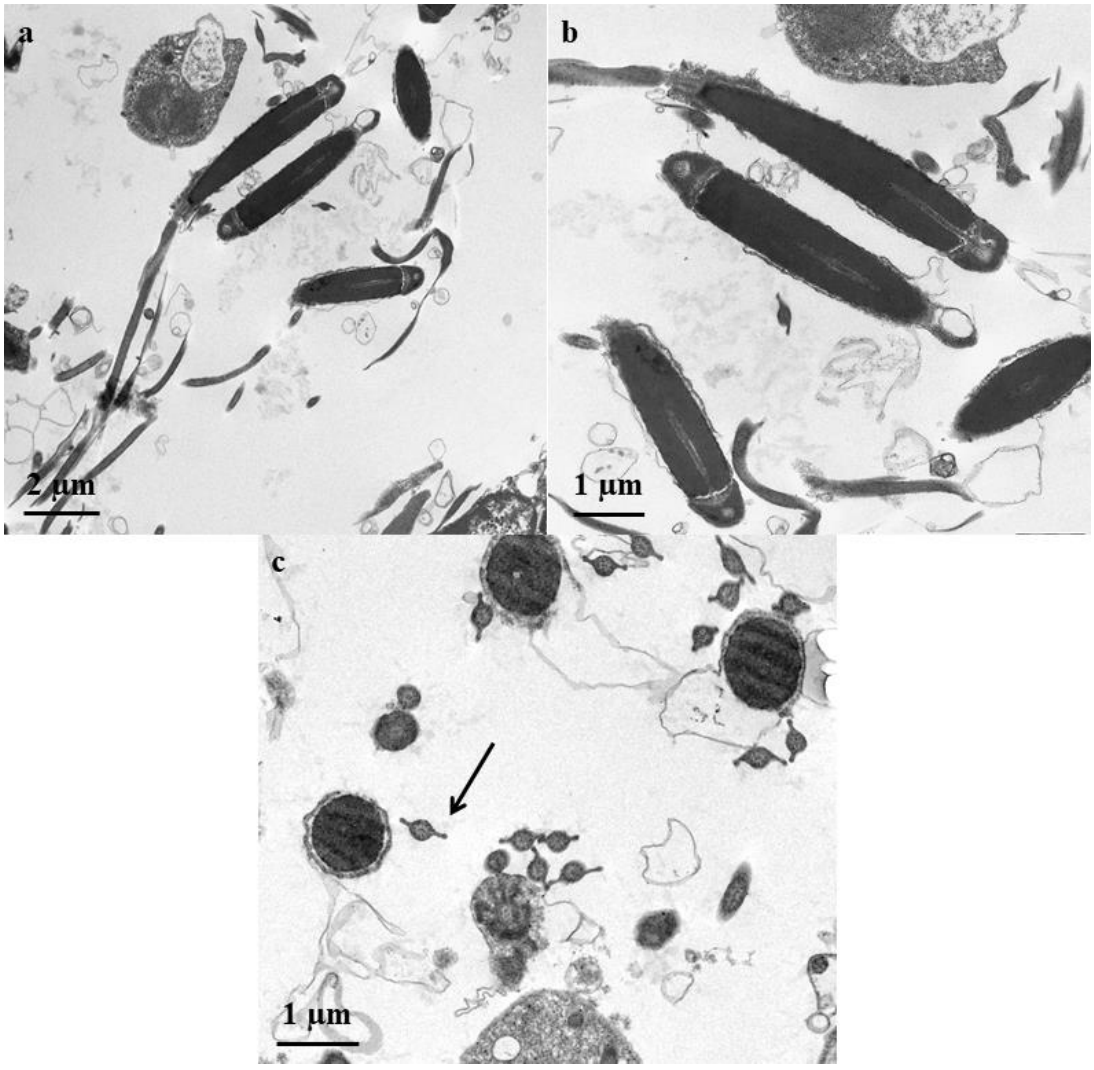

Figure 3.7 *Platynereis massiliensis*-like from the Castello Aragonese S3/S2 vent areas (Ischia Island) mature spermatozoa TEM pictures: (a-b) spermatozoa with elongated heads; (c) section off flagella with lateral projections (arrow).

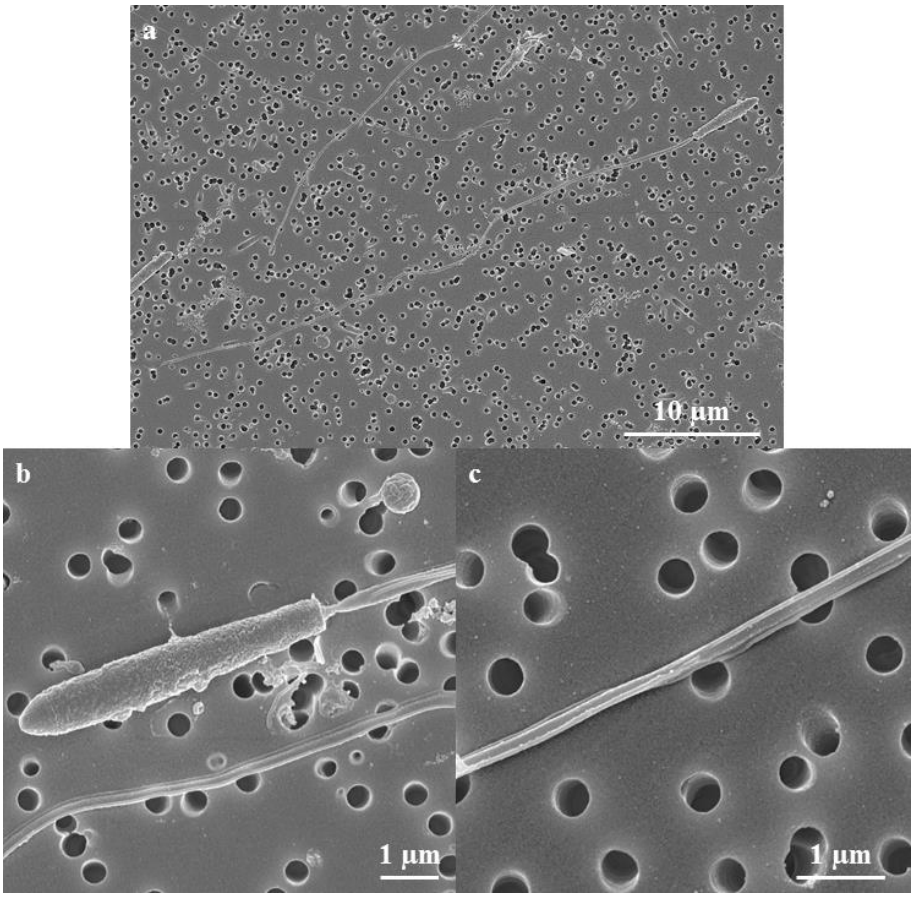

Figure 3.8 *Platynereis massiliensis*-like from the Castello Aragonese S3/S2 vent areas (Ischia Island) mature spermatozoa SEM pictures: (a) whole spermatozoon; (b) sperm head; (c) flagellum with lateral projections.

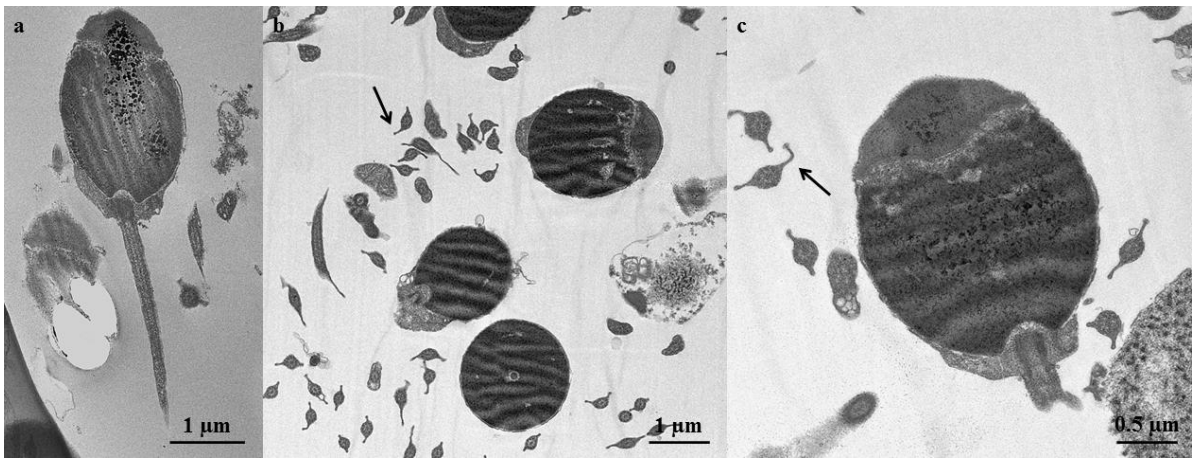

Figure 3.9 *Platynereis dumerilii*-like from Sant'Anna (Ischia Island) and Santa Caterina (Lecce) mature spermatozoa TEM pictures: (a) sperm section; (b-c) sperm heads and flagella sections with lateral projections (arrows).

3.4 Discussion

Reproductive biology observations highlight the occurrence of two different strategies: a gonochoric reproduction with epitokous transformation and gametes broadcasting habit, typical of *Platynereis dumerilii*; a protandric hermaphrodite habit with eggs brooding inside the tube, typical of *P. massiliensis* (Hauenschild 1951, Schneider et al. 1922, Fischer et al. 2010). The brooding habit is the dominant strategy in the three vent systems of Ischia, Vulcano and Panarea Islands, while the broadcasting epitokous habit mainly occurs in normal pH areas (e.g. Sant'Anna and La Spezia). Several hypothesis may explain this peculiar distribution for the reproductive habits.

As for the Castello acidified area, this result is consistent with previous observations. In fact Lucey et al. (2015), focusing on polychaetes as one of the most abundant taxonomic groups in the CO_2 vent system of Ischia Island, highlighted that the brooding *Platynereis massiliensis* showed a marked preference in colonizing the naturally acidified habitat of the Castello, while the broadcasting *P. dumerilii* was more abundant in the nearest normal pH areas. The authors asserted that species with life history strategies linked to parental care are favoured and more protected in acidified conditions, compared to their broadcast spawning relatives with pelagic larval development (Lucey et al. 2015). Our results allow hypothesizing of a pH-driven selection of the brooding *Platynereis massiliensis* in the vent systems of Ischia and Vulcano, as previously observed at the Castello vents (Calosi et al. 2013b). An evolutionary selection of tolerant phenotypes to OA is supported by the recognized ability of polychaetes to adapt to chronically disturbed habitats (Calosi et al. 2013b, Lucey et al. 2015).

Alternatively, the CO_2-dominating brooding *Platynereis massiliensis* could be favoured by the unique chemical conditions of the vent area, which induce a 'chemical island' effect, acting as a sort of barrier for pelagic larval stages greatly reducing their settlement and recruitment (Lucey et al. 2015). The CO_2 vent systems can be considered as isolated habitats in which the brooding dispersal capacity is only theoretically, but not actually limited by the low mobility of the early developmental phases. As demonstrated by the 'Rockall paradox' for the brooding and broadcasting gastropod species, *Littorina saxatilis* and *L. littorea*, isolated habitats (i.e. islands) are void of any

pelagic broadcast spawing invertebrates as their larvae may not be able to find, settle and reproduce in distant places (Johannesson 1988).

The *Platynereis massiliensis* success in low pH habitats may also be due to the winning reproductive biology strategy in stressful conditions. Infact, this species lives inside tubes attached to algae, which produce oxygen throughout photosynthesis, buffering the negative effects of high CO_2 content and low pH conditions. In addition, the microhabitat conditions and ventilation of the eggs inside the tubes could facilitate the embryos' survival and the juveniles' semi-direct developments. A similar buffering effect of algae has already been shown for the blue mussel *Mytilus edulis*, whose calcification is facilitated even under acidified conditions thanks to the carbonate system fluctuations produced by the diurnal photosynthesis/respiration cycles of the brown alga *Fucus vesiculosus* and the sea grass *Zostera marina* (Wahl et al. 2017). The small spirorbid polychaete species *Spirorbis spirorbis* (Serpulidae), which settled on the brown alga *Fucus serratus*, shows higher growth rates in low pH conditions when the thalli are exposed to light and therefore could photosynthesize, compared to specimens which lived in thalli under dark conditions (no photosynthesis allowed) (Saderne and Wahl 2012). Spirorbid polychaetes are quite small in size (a few mm) so that the effect of algal photosynthesis could be highly influential. Although *Platynereis massiliensis* is of a larger size (up to 50 mm), the positive influence of algal buffering via photosynthesis can be hypothesized due to the highly dense cover and biomass of macroalgae which characterize the vent areas considered, at least for the Castello (Ricevuto et al. 2015b, Gambi et al. 2016b) and Vulcano systems (Vizzini et al 2017). The winning life history strategy of *Platynereis massiliensis* in disturbed conditions could be translated into its success for space and food competition against the broadcasting *P. dumerilii*.

Interestingly, the acidified site of Panarea, as well as the normal pH sites of Ponente Bay and Santa Caterina, is characterized by the coexistence of specimens with both reproductive strategies, which means both species co-occurred in the same areas. Panarea Island and Ponente Bay are dominated by brooding specimens, and only a few individuals underwent the unique sexual metamorphosis of *P. dumerilii* (¼ and $^1/_7$, respectively). Conversely, *Platynereis dumerilii* heteronereis stage is the most frequent in Santa Caterina. These results confirm the sympatric nature of the sibling species characterized by different reproductive strategies, and it is unsurprising to find the

brooding species outside vents as well as the occurrence of putative *Platynereis dumerilii* in some of the acidified areas, although with limited numbers.

The experiments of cross-breeding enable hypothesis about the reproductive isolation between vent populations due to the brooding habit and the elevated geographical distances among sampling sites, at least between Ischia and the Aeolian islands (Vulcano and Panarea) (approx. 450 km). Previous research aimed at characterizing closely releated polychaete species has encompasses various combination of study approaches, but only few studies of species differentiation are backed up with interbreeding studies that are fundamental for sibling species identification. Laboratory cross-breeding experiments have lead to the identification of morphologically similar but reproductively isolated species of the genus *Ophryotrocha* (Åkesson 1984), of a separate sibling species within the *Capitella capitata* complex (Gamenick et al. 1998), and of three reproductively and genetically distinct species of the *Polydora cornuta* complex in North America (Rice et al. 2008).

Sperm morphology has been widely regarded as a valid means to separate one polychaete sibling species from another (Franzén 1956, Rice and Simon 1980, Olive 1983, Pfannenstiel and Gruenig 1990). The mature spermatozoa morphology observed in both *Platynereis* species is consistent with previous studies, with the exception of *P. massiliensis* sperm size, smaller than the Lücht and Pfannenstiel (1989) description (10 μm in length and 1.5 μm in diameter) (no information on *P. dumerilii* sperm size is available). TEM and SEM observations clarify the sperm morphology and confirm the occurrence of differences among the broadcasting *Platynereis dumerilii* and the brooding *P. massiliensis*. As discussed by Pfannenstiel et al. (1987), the sperm morphology of *Platynereis dumerilii* can be considered as the primitive type (ect-aquasperm type, according to Jamieson and Rouse 1989), which favours the free-spawning gamete release into the water column and external fertilization. On the other hand, *Platynereis massiliensis* sperms can be identified as the aberrant type (ent-aquasperm type, according to Jamieson and Rouse 1989) useful in case of pseudocopulation inside of brooding tubes (Lücht and Pfannenstiel 1989) or for fertilization in jelly masses and inside body masses (Jamieson and Rouse 1989). Therefore, spermatozoa morphology is highly consistent with reproductive habit in both sibling *Platynereis* species.

Chapter 4

Genetic differentiation of *Platynereis dumerilii* and *P. massiliensis* sibling complexes in relation to Ocean Acidification

4.1 Introduction

The continued release of fossil-fuel derived CO_2 into the atmosphere is causing an alteration of the seawater carbonate chemistry, at geologically unprecedented rates (Doney and Schimel 2007). It is predicted to produce a pH drop off of 0.3-0.5 units by the year 2100 (Caldeira and Wickett 2003, Feely et al. 2004). The potential impact of this phenomenon, known as ocean acidification (OA), has been deeply investigated on the most vulnerable species, mainly marine calcifiers organisms (e.g. corals, molluscs, coralline algae, etc.) (Orr et al. 2005, Fabry et al. 2008). Much less information is available on non-calcifying organisms in which OA could anyway affect species at physiological or genetic level, with negative consequences from the single organism up to whole communities, affecting biodiversity and trophic structure (Kroeker et al. 2011, Calosi et al. 2013b, Harvey et al. 2014). Marine areas with volcanic carbon dioxide bubbling (CO_2 vent systems), cause the natural acidification of surrounding waters and are used as model natural laboratories to investigate both short and long-term effects of OA on benthic biodiversity (Hall-Spencer et al. 2008, Fabricius et al. 2011). One of the world's most famous and well-studied natural laboratory is the volcanic CO_2 vent system of the Castello Aragonese islet in Ischia (Italy) (Hall-Spencer et al. 2008). In the vegetated rocky reefs, as well as in the *Posidonia* meadow of this naturally acidified site, annelid polychaetes represent one of the dominant taxa of the benthic community (Kroeker et al. 2011, Garrard et al. 2014, Gambi et al. 2016b). The polychaete model species *Platynereis dumerilii* (Audouin and Milne-Edwards, 1834) was identified as a tolerant organism to OA by Calosi et al. (2013b) due to its relatively high abundance in the most acidified sites of Castello Aragonese (Ricevuto et al. 2014). *Platynereis dumerilii* is a non-calcifying annelid worm of the family Nereididae. In the adult phase it is a benthic meso-herbivore species (Gambi et al. 2000; Ricevuto et al. 2015b), with separate sexes and a sexual maturation which implies drastic morphological changes in a so called 'heteronereis' form with pelagic lifestyle (epitokous transformation) (Fischer et al. 2010). The gamete fertilization occurs in the water column once they have been released by males and females swarming at night. After gamete release, both parents die and the larvae go through a subsequent planktonic developmental phase (Fischer and Dorresteijn 2004). *Platynereis dumerilii* has a single sibling species documented in the current literature, *P. massiliensis* (Moquin-Tandon, 1869), with a morphologically-

indistinguishable sexually immature adult stage, although characterized by completely different life history traits, reproductive biology and gamete morphology (Schneider et al. 1992, Hauenschild 1951, Lücht and Pfannenstiel 1989). *Platynereis massiliensis* is, in fact, a protandric hermaphrodite species with no epitokous transformation. It is a brooder with the female that lays eggs inside the tube to be fertilized by the male and then dies; later the male continues ventilating the developing embryos (Schneider et al. 1992).

Genetic preliminary results (Calosi et al. 2013b) (see Fig. 1.7, Chapter 1) and reproductive biology observations (Lucey et al. 2015) demonstrated that the *Platynereis* population, thriving inside the acidified zones of the CO_2 vent system of Castello Aragonese, belonged to the neglected sympatric sibling species *P. massiliensis* (Lucey et al. 2015, Valvassori et al. 2015). Genetic results highlighted that various control areas (normal pH), outside Castello Aragonese vents, are dominated by *Platynereis dumerilii* with a proportion compared to the sibling of 15:1. Conversely, *Platynereis massiliensis* prevailed in the acidified sites in the proportion of 10:1 over *P. dumerilii* (Lucey et al. 2015). The predominance of the brooding *Platynereis massiliensis* in acidified areas seemed not to be directly correlated with the low pH effect in the cryptic speciation from the broadcasting *P. dumerilii*, but with a winning reproductive strategy in stressful conditions, such as OA (Lucey et al. 2015). In a more recent study (Wäge et al. 2017), a phylogeographic approach coupled with reproductive biology/life history observations was used to investigate, on a larger spatial-scale, the possible selective effect of low pH conditions on genotypes of either *Platynereis dumerilii* or *P. massiliensis* and their geographical distribution. The studied *Platynereis* spp. specimens were collected from two different CO_2 vent systems (Ischia and Vulcano islands – Southern Tyrrhenian Sea, Italy), as well as various non-acidified sites from the Mediterranean Sea and the Atlantic Ocean. The authors highlighted the occurrence of four different clades: two of them clustered specimens from the acidified areas of Ischia (clade 1) and Vulcano (clade 2) islands showing the same parental care strategy, congruent with the brooding reproductive habit of *Platynereis massiliensis* (Helm et al. 2014) (Fig. 4.1); clades 3 and 4 clustered individuals mainly from normal pH areas and *P. dumerilii* COI reference sequence (Fig. 4.1). The significant mean genetic distances between clades (clades 1 *vs* 2 25.5%; clades 3 *vs* 4 22%) allow the authors to hypothesize that both *Platynereis massiliensis* and *P. dumerilii* represent complexes of sibling species (Wäge et al. 2017).

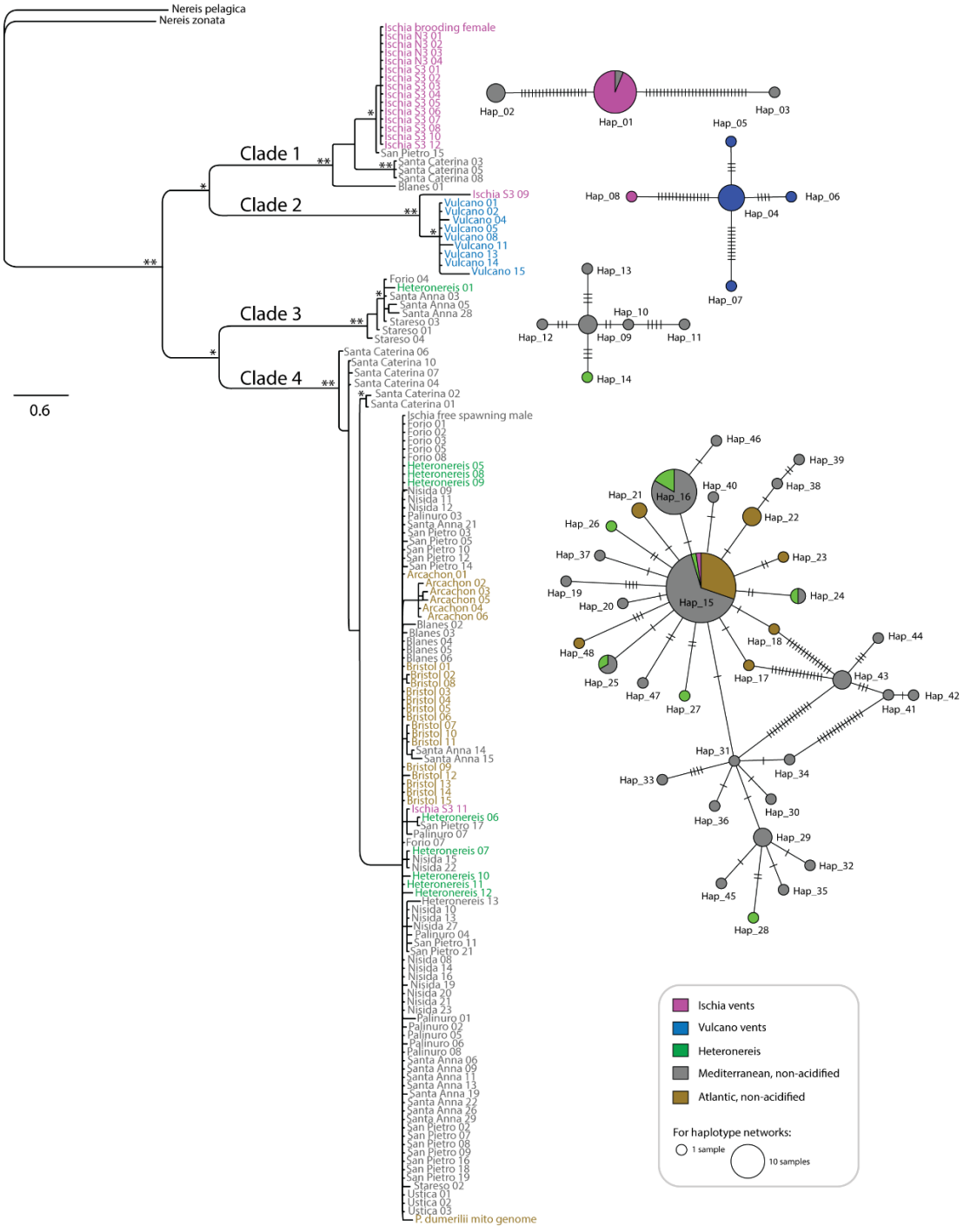

Figure 4.1 50% Majority rule consensus tree based on Bayesian inference. Inference and haplotype networks for each of the four clades. Asterisks at nodes indicate posterior probability (**: 100%; *: >90%; branch support values < 90% not shown). The size of the circles in the haplotype networks indicates the number of sequenced individuals with this haplotype. Hash marks on the connecting lines indicate the number of mutational steps between two haplotypes. The colours identify: Ischia vents specimens in pink, Vulcano vents specimens in blue, Heteronereis specimens in green, Mediterranean and Atlantic specimens from non-acidified sites in grey and brown, respectively (from Wäge et al. 2017).

Through the amplification of the COI mitochondrial gene, the present work aimed to perform a phylogeographic analysis on *Platynereis* spp. specimens collected from four naturally CO_2 vent systems and four control areas located along the Italian coasts. Compared to the Wäge et al. (2017) paper, the present study is based on the COI amplification of 339 new *Platynereis* spp. samples collected from shared sampling sites (acidified: Castello Aragonese, Levante Bay; ambient pH: Sant'Anna, Santa Caterina) and new ones (acidified: Panarea Hot/Cold points and Bottaro crater; ambient pH: Blue Bay, Ponente Bay). The results of this study, coupled with the Wäge et al. (2017) outcomes, will provide important information that will allow researchers to better understand and explore the complicated population genetic structure of *Platynereis* and the occurrence of sibling species or complexes, presumably characterized by different reproductive modes. They will also enable to find more signs to support the hypothesis that brooding specimens, putatively belonging to the *Platynereis massiliensis* complex, represent the selected or favoured phenotype/genotype in conditions of OA.

4.2 Materials and methods

4.2.1 Collection of specimens and sample preparation

Platynereis spp. specimens were collected in eight different sampling sites over the years 2015-2016, mainly during April and May (Tab. 4.1). The selected sampling sites were four different naturally acidified systems (Castello Aragonese – Ischia Island, Levante Bay – Vulcano Island, Ditella beach Hot/Cold points and Bottaro crater – Panarea Island) and four normal pH areas (Blue Bay – La Spezia, Sant'Anna – Ischia Island, Ponente Bay – Vulcano Island, Santa Caterina – Lecce) (see Table 4.1). *Platynereis* spp. specimens were collected in macroalgae, mainly *Halopteris scoparia*, *Dictyota* spp., and *Cladophora* spp. in Ischia; *Cystoseira compressa* and *Dictyota dichotoma* in Vulcano; *Cystoseira foeniculacea* and *C. brachycarpa* var. *balearica* in Panarea. Macroalgae were sampled by hand (SCUBA diving or snorkelling) in shallow water vegetated rocky reef habitats at approximately 0.5-2.5 m depth in each site, except in Panarea where the acidified areas are located at 10-11 m depth. A standardized qualitative sampling protocol was followed and three cotton fabric bags (25 x 25 cm) of thalli (approx. 500 g w.w. each) were sampled from each sampling site. Target species were sorted shaking the algae inside plastic trays, and were immediately transferred into separate vials (one for each individual) and fixed in 95% ethanol (and then preserved at +4 °C) or, when it was possible, frozen at -80 °C for genetic analyses.

Table 4.1 Summary table of the eight selected sampling sites with relative geographical coordinates and pH conditions.

Sampling site	Coordinates	pH
Blue Bay, La Spezia	44°04'58.08"N, 9°53'02.52"E	Normal
Castello Aragonese, Ischia Island	40°43'50.80"N, 13°57'47.80"E	Acidified
Sant'Anna, Ischia Island	40°43'34.64"N, 13°57'36.33"E	Normal
Ditella Hot/Cold points, Panarea Island	38°38'21.16"N, 15°4'42.84"E	Acidified
Bottaro crater, Panarea Island	38°38'15.73"N, 15°6'36.13"E	Acidified
Levante Bay, Vulcano Island	38°25'10.37"N, 14°57'41.11"E	Acidified
Ponente Bay, Vulcano Island	38°25'15.52"N, 14°57'17.72"E	Normal
Santa Caterina, Lecce	40°08'20.70"N, 17°58'47.80"E	Normal

4.2.2 Sample processing: gDNA extraction and COI PCR amplification

Total genomic DNA (gDNA) was extracted using the modified cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle 1987, 1990). The detailed protocol is described in the Appendix 1. gDNA quality and size were estimated by 1% agarose gel electrophoresis in 0.5X TBE (5X TBE: 1.1 M Tris; 900 mM Borate; 25 mM EDTA; pH 8.3). The gDNA fragments of the mitochondrial cytochrome *c* oxidase subunit I (COI, approx. 600 bp) were amplified via polymerase chain reaction (PCR) using primers as described by Folmer et al. (1994): LCO-1490 5'-GGTCAACAAATCATAAAGATATTGG-3'; HCO-2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'. PCR amplification were performed in a Thermal cycler in 20 µL total reaction volume containing: 1 µL of gDNA (concentrated or 10-100 times diluted), 2 µL 10X PCR reaction buffer (Roche), 2 µL dNTPs 2 mM (Roche), 0.8 µL of each forward and reverse primer (10 pmol/ µL), 0.5 µL Taq DNA Polymerase 5U/µL (Roche) and brought to the final volume with Milli-Q water. The thermocycling profile consisted in an initial denaturation step of 3 min at 95 °C followed by 35 cycles of 60 s at 95 °C (denaturation), 60 s at 40 °C (annealing) and 90 s at 72 °C (extension), with a final extension step of 7 min at 72 °C. PCR products were screened for correct length by UV fluorescence on agarose gel (1.5% agarose w/v, 0.5X TBE) stained with ethidium bromide and then purified with GenElute™ Gel Extraction Kit by SIGMA following the manufacturer's instructions. The purified PCR products were sequenced in both strands, using amplification primers on 48 capillaries Applied Biosystems (Life Technologies) 3730 DNA Analyzer using Big Dye®-terminator chemistry at the Molecular Biology and Sequencing facility of the Stazione Zoologica Anton Dohrn, Naples.

A total of 339 *Platynereis* spp. specimens were analysed: 50 individuals from Blue Bay; 50 individuals from Castello Aragonese and Sant'Anna, respectively; 50 from Levante Bay and Ponente Bay, respectively; 49 individuals from Panarea Island (30 from Hot/Cold points and 19 from Bottaro crater) and 40 individuals from Santa Caterina.

4.2.3 Data analysis

Sequence identities were verified using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained raw sequences were checked using Chromas v 2.6.2 (Technekysium Pty Ltd, Queensland, Australia), aligned with BioEdit 7.2.5 (Hall 1999) using the CLUSTAL W Multiple Alignment option with default settings (Thompson et al. 1994), and finally adjusted by eye. Phylogenetic analyses were inferred by using Maximum Likelihood, Minimum Evolution and Maximum Parsimony methods based on the Kimura-2-Parameter model with Bootstrap method (1,000 bootstrap replications) in MEGA v 6 (Tamura et al. 2013). The average genetic distances among clades were also calculated using the Kimura-2-Parameter model in MEGA v 6. Bayesian inference (BI) was performed with MrBayes v 3.2 (Ronquist et al. 2012) using two runs with four Metropolis Coupled Markov Chains Monte Carlo (MCMCMC) each for 10,000,000 generations under a General Time Reversible Model plus Gamma, with the first 2,500,000 generations discarded as burn-in. Trees were sampled every 1,000 generations; resulting p-files were examined in Tracer v 1.6 (Rambaut et al. 2014) to evaluate convergence and to ensure sufficient burn-in for the trees and finally consensus tree was generated and visualized with FigTree v 1.4.2. The COI sequences from the complete mitochondrial genome of *Platynereis dumerilii* (Boore and Brown 2000, Genbank accession AF178678), 'Ischia free spawning male' (Lucey et al. 2015, Genbank accession KP127954) and 'Ischia brooding female' (Lucey et al. 2015, Genbank accession KP127953) were included. *Nereis pelagica* (Genbank accession GU672554) and *N. zonata* (HQ024403) were used as outgroup.

Genetic diversity measures including number of haplotypes (H), haplotype diversity (Hd), number of polymorphic sites (S), nucleotide diversity (π) as well as the mean number of pairwise differences (θ) were calculated using Arlequin 3.5 (Excoffier et al. 2010).

4.3 Results

The phylogenetic tree (Fig. 4.2) shows that *Platynereis* spp. specimens cluster in four different clades.

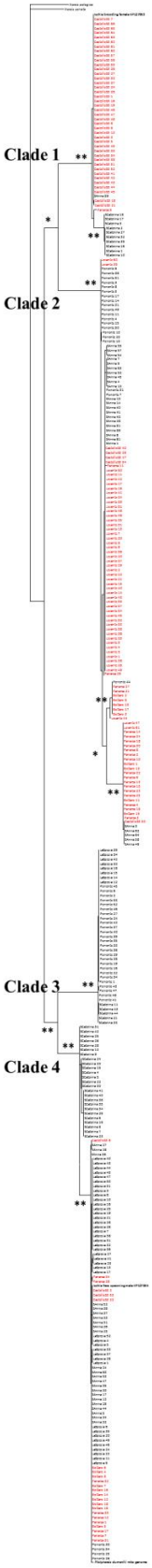

Figure 4.2 50% Majority rule consensus tree based on Bayesian inference. Asterisks at nodes indicate posterior probability (**: 100%; *: > 90%; branch support values < 90% not shown). The samples highlighted in red were collected in acidified sampling sites.

Clade 1 is mostly composed of individuals from the acidified area of Ischia (Castello Aragonese), with 41 individuals grouped with only one specimen from the Sant'Anna control area (SAnna_59) (Fig. 4.3). These specimens cluster together with the 'Ischia brooding female' COI mitochondrial gene sequence. The same clade includes a Santa Caterina branch, with 10 specimens plus one individual from the Hot/Cold points of Panarea Island (Panarea_9) (Fig. 4.3).

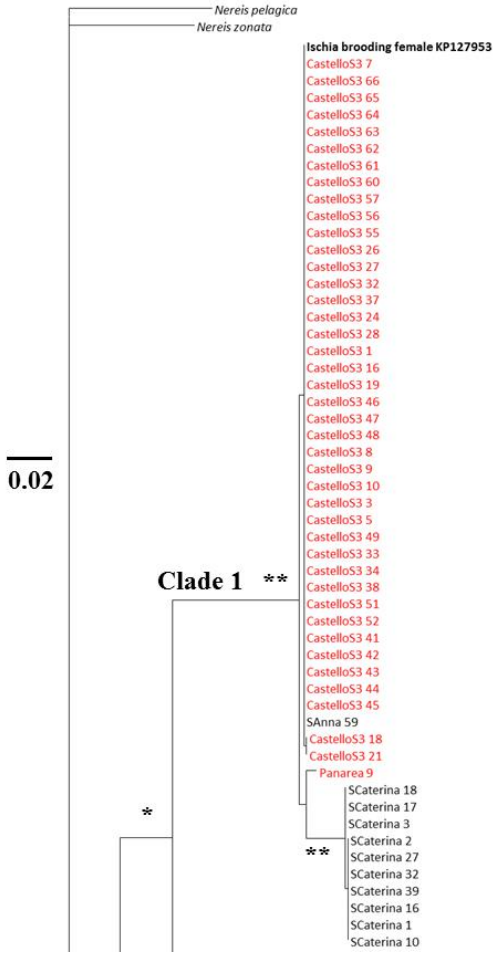

Figure 4.3 *Clade 1*: 50% Majority rule consensus tree based on Bayesian inference. Asterisks at nodes indicate posterior probability (**: 100%; *: > 90%; branch support values < 90% not shown). The samples highlighted in red were collected in acidified sampling sites.

Clade 2 is a large clade that branches out in several smaller groups with: all 50 specimens from the vent area of Vulcano Island (Levante Bay), 29 individuals from the acidified area of Panarea Island that cluster into two smaller branches (20 from Hot/Cold points and 9 from Bottaro crater), 26 from Sant'Anna, 20 from Ponente Bay (Vulcano Island) and only 5 individuals from the Castello Aragonese vent system (Fig. 4.4).

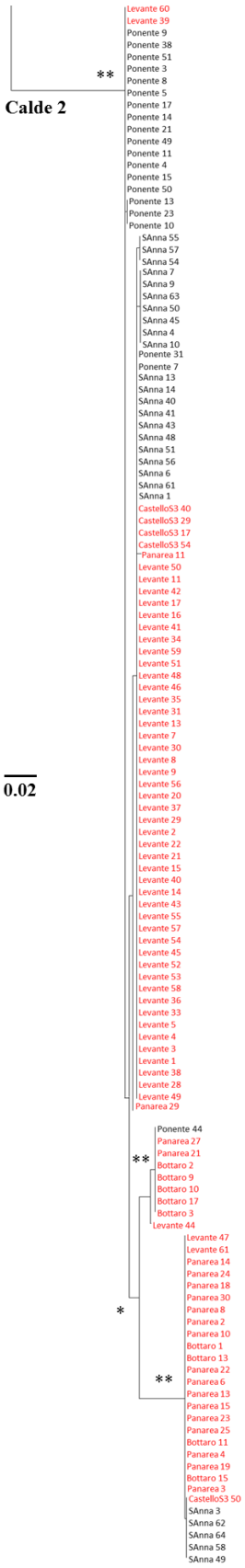

Figure 4.4 *Clade 2*: 50% Majority rule consensus tree based on Bayesian inference. Asterisks at nodes indicate posterior probability (**: 100%; *: > 90%; branch support values < 90% not shown). The samples highlighted in red were collected in acidified sampling sites.

Clade 3 includes only specimens from normal pH areas: 26 individuals collected in Ponente Bay, 8 from Blue Bay and 5 specimens from Santa Caterina (Fig. 4.5).

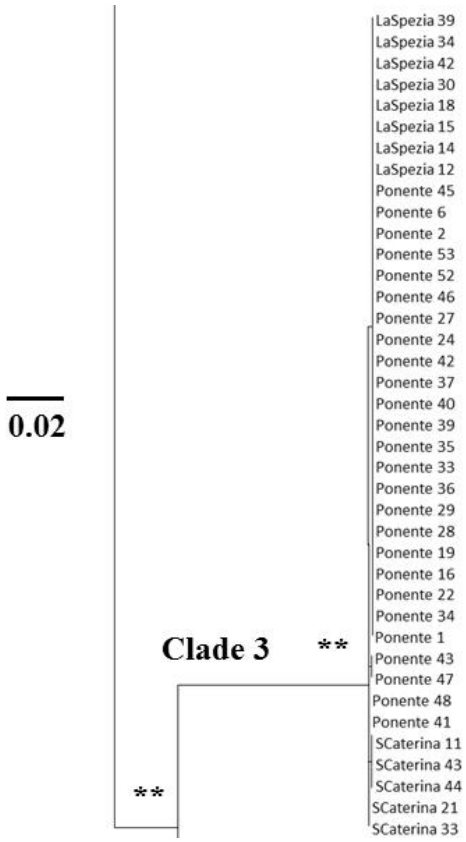

Figure 4.5 *Clade 3*: 50% Majority rule consensus tree based on Bayesian inference. Asterisks at nodes indicate posterior probability (**: 100%; *: > 90%; branch support values < 90% not shown).

Clade 4 is mainly composed of specimens from non-acidified areas with 42 individuals from Blue Bay, 23 collected in Sant'Anna and 4 individuals from Ponente Bay (Fig. 4.6). Some specimens from the vent systems of both Panarea (19 out of 49; 9 from Hot/Cold points and 10 from Bottaro crater) and Ischia (4 out of 50) islands are also included in this clade (Fig. 4.6). These sequences cluster together with both COI sequences from the *Platynereis dumerilii* complete mitochondrial genome and COI 'Ischia free spawning male' sequence. A detached group composed of 25 sequences of Santa Caterina is inside the same clade (Fig. 4.6).

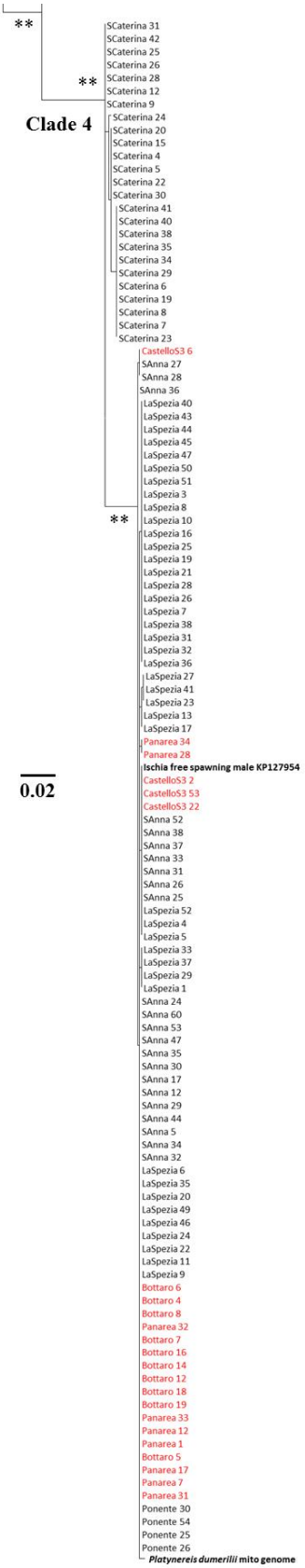

Figure 4.6 *Clade 4*: 50% Majority rule consensus tree based on Bayesian inference. Asterisks at nodes indicate posterior probability (**: 100%; *: > 90%; branch support values < 90% not shown). The samples highlighted in red were collected in acidified sampling sites.

The phylogenetic analyses calculated the average genetic distances (Kimura-2-Parameter model) of 19.9% between clade 1 and clade 2, and 16.8% between clade 3 and 4. The genetic composition of each sampling site is depicted in the Fig. 4.7 and it is summarized in Table 4.2.

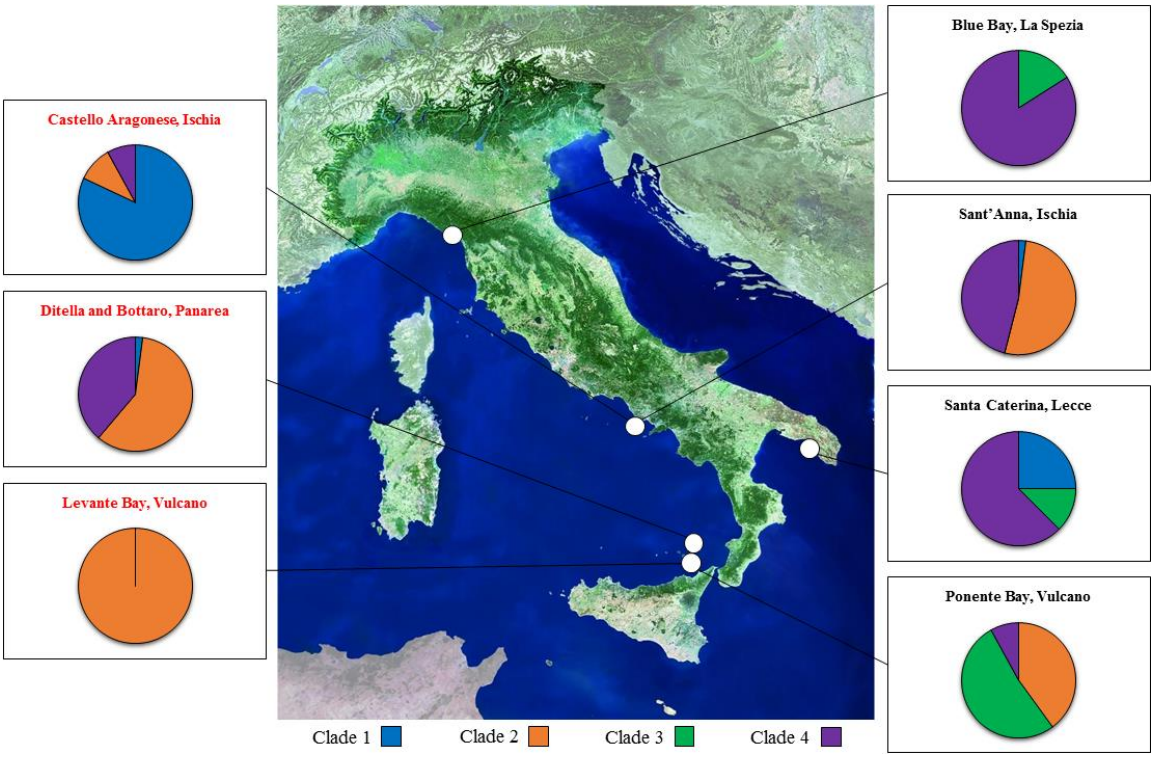

Figure 4.7 Pie charts of clade composition of each sampling site.

Table 4.2 Percentage of individuals belonging to each clade in each sampling site.

Sampling site	Clade 1	Clade 2	Clade 3	Clade 4
Blue Bay, La Spezia	-	-	16%	84%
Castello Aragonese, Ischia	82%	10%	-	8%
Sant'Anna, Ischia	2%	52%	-	46%
Hot/Cold points and Bottaro crater, Panarea	2%	59%	-	39%
Levante Bay, Vulcano	-	100%	-	-
Ponente Bay, Vulcano	8%	40%	52%	-
Santa Caterina, Lecce	25%	-	13%	63%

The results of the genetic diversity measures, comparing sampling sites and clades, are summarized in Tables 4.3 and 4.4:

Table 4.3 Genetic diversity measures considering the different sampling sites/geographical areas. H: numbers of haplotypes; Hd: haplotype diversity; S: number of polymorphic sites; π: nucleotide diversity; θ: mean number of pairwise differences.

	H	**Hd**	**S**	**π**	**θ**
Blue Bay, La Spezia	7	0.766	92	0.044	25.590
Castello Aragonese, Ischia	6	0.387	161	0.059	34.424
Sant'Anna, Ischia	9	0.846	163	0.103	59.675
Hot/Cold points and Bottaro crater, Panarea	7	0.704	163	0.103	60.069
Levante Bay, Vulcano	4	0.190	40	0.006	3.588
Ponente Bay, Vulcano	8	0.727	156	0.117	68.051
Santa Caterina, Lecce	8	0.847	143	0.096	55.781

Table 4.4 Genetic diversity measures considering the different clades. H: numbers of haplotypes; Hd: haplotype diversity; S: number of polymorphic sites; π: nucleotide diversity; θ: mean number of pairwise differences.

	H	**Hd**	**S**	**π**	**θ**
Clade 1	5	0.416	26	0.012	6.816
Clade 2	11	0.724	45	0.024	13.965
Clade 3	4	0.399	3	0.001	0.610
Clade 4	13	0.809	25	0.011	6.594

4.4 Discussion

This phylogeographic study reveals several interesting insights. According to the tree (Fig. 4.2), clade 1 is mainly composed of specimens collected from acidified areas of Ischia that clustered together with the brooding female sequenced in Lucey et al. (2015). Two thirds of the total sequences included in clade 2 were collected in three different acidified areas. It is interesting to note that the Vulcano Levante Bay population is entirely grouped in this clade (2), together with most of the specimens from Panarea. This is unsurprising considering that these two islands belong to the Aeolian Archipelago and are located approximately 20 miles apart. As reported in Chapter 3, the majority of populations grouped in these two clades show the brooding behaviour typical of *Platynereis massiliensis* (Hauenschild 1951, Schneider et al. 1992, Helm et al. 2014). The genetic results and the congruence with the reproductive observations suggest that specimens grouped in clades 1 or 2 belonged to the neglected sibling *Platynereis massiliensis* (Valvassori et al. 2015). Therefore, to verify whether these clades truly represent the originally described species, it would be advisable to compare the sequences of both clades with those obtained from individuals of *Platynereis massiliensis* collected in its type locality (Marseille, France). A small fraction of clades 1 and 2 specimens were collected in normal pH sampling sites ($^1/_5$ and $^1/_3$, respectively); it is not surprising that brooding specimens are able to survive even outside the vent systems, in unstressed conditions. Clade 3 is entirely composed of individuals sampled in normal pH areas. Clade 4 mainly includes specimens from normal pH sites and they can be considered as putative *Platynereis dumerilii* due to the inclusion of both reference sequence of the *P. dumerilii* mitochondrial genome and the COI of an 'Ischia free spawning male' sequence in this cluster. However, the geographical origin of the specimen used to generate the mitochondrial genome is unknown (Jeff Boore, pers. comm.) and, considering the occurrence of multiple cryptic lineages, a species misidentification cannot be excluded. To solve this problem, the comparison with the COI sequence of *Platynereis dumerilii* specimens from its type locality (La Rochelle, northern France) would be necessary. Clade 4 groups also few individuals from the Castello Aragonese vent population likely due to the fact that acidified and control sites in Ischia Island are only 600 m apart, and that adult specimens of *Platynereis dumerilii* may thrive under low pH conditions. Whether individuals of the free spawner

Platynereis dumerilii successfully reproduce in acidified conditions remains to be investigated, although some epitokous swimming specimens were sampled at night in the acidified area of Castello Aragonese (24 May 2011, Hardege J., Larsson T. and Gambi M.C. personal observations; Wäge et al. 2017). The samples sequences from both Panarea acidified sites, Hot/Cold points and Bottaro crater, cluster together on both clades 2 and 4 without showing genetic differentiation based on sampling site. This is likely due to the relative proximity between the two populations (approx. 1.5 km apart) and the similar environmental conditions (and pH levels) recorded in both sampling stations. As highlighted also in Wäge et al. (2017), Santa Caterina samples create a sub-clade, slightly differentiated from the other sequences in both clades 1 and 4. This might be due to the accumulation of genetic mutation caused by the distance between Tyrrhenian and Ionian populations, creating a geographical barrier to the gene flow. Increased environmental distance may result in increased genetic distance as individuals or populations adapt to different environmental factors in different regions (Frankham et al. 2002). A similar phenomenon has already been observed for Mediterranean marine organisms, which are more motile than polychaetes, such as the red mullet *Mullus barbatus* (Maggio et al. 2009) and the dinoflagellate, *Alexandrium minutum* (Casabianca et al. 2011).

The clades subdivision as well as the significant average genetic distances calculated between clades (clades 1 *vs* 2 19.9%; clades 3 *vs* 4 16.8%) are coherent with the previous observations of Wäge et al. (2017), and validate the hypothesis that both *Platynereis massiliensis* and *P. dumerilii* represent complexes of sibling species. Similar or even lower percentages of mean genetic distances have been sufficient to distinguish different species that are morphologically similar. A genetic distance of 11.7% in the mitochondrial COI was enough to identify two populations of the annelids *Vigtorniella* from the shallow-water Atlantic and the deep-sea Pacific as different species (Wiklund et al. 2009); as well as differences of 5-24.8% in COI among oligochaete congeneric species (Bely and Wray 2004), and 7-14% in COI between sister species of siboglinid polychaetes (Chevaldonné et al. 2002).

Furthermore, the sequences distribution in the COI tree is consistent with the reproductive biology observations, with the only exception of some individuals from Castello Aragonese and Sant'Anna (see Chapter 3), confirming the statement that a brooding reproductive strategy seemed to be favoured under OA conditions (Lucey et

al. 2015, Wäge et al. 2017), at least for polychaetes (Gambi et al. 2016b). The clades subdivision is not correlated with the geographical location or the latitudinal gradient, with the exception of the Levante Bay vent population, which is entirely composed of individuals belonging to clade 2 (Fig. 4.7). Furthermore, clade 3 is the only one that includes all individuals sampled in normal/control pH areas (Blue Bay – La Spezia, Ponente Bay – Vulcano Island, Santa Caterina – Lecce). The two sibling complexes seem to coexist in Sant'Anna, Panarea, Ponente Bay and Santa Caterina. The genetic diversity measurements confirm that the Levante Bay population is characterized by a single species with a low level of polymorphisms; the same situation is also observed for clade 3. As far as the other populations and clades, they appeared characterized by higher levels of polymorphisms.

The phenomenon of sibling/cryptic speciation is particularly common within model polychaetes species (Grassle and Grassle 1976, Virgilio et al. 2005, Burlinson and Lawrence 2007, Durou et al. 2007, Vázquez-Núñez et al. 2007, Dean 2008, Blake et al. 2009). A proper identification of sibling species of the widely used model organism *Platynereis dumerilii* plays a fundamental role and could have several implications in different research fields. It is unsurprising that brooding species, with direct/semi-direct development and low dispersal rates, show isolated populations and increased genetic diversity (Palumbi 1994). Two different forms of the nereidid polychaete *Hediste japonica*, which differ in life-history strategies (small-eggs form *vs* large-eggs form), had different rates of gene flow between populations (Sato and Tsuchiya 1991, Sato and Masuda 1997). More frequent gene flow was observed in the free-swimming larvae of the smaller-eggs form that easily migrate; on the contrary, the direct development of the larger-eggs form into benthic juveniles resulted in a limited gene flow between populations (Sato and Tsuchiya 1991, Sato and Masuda 1997). Low gene flow levels and regional phylogeographic fragmentation have also been observed in two direct developer marine isopods species (Teske et al. 2007) and in the direct developer polychaete species *Neantes acuminata* (Reish et al. 2014). The same explanation can clarify the genetic complexity of *P. massiliensis* clades (1 and 2).

A selective pressure might also derive from the extreme conditions of CO_2 vent systems; even if it is not directly responsible for the cryptic speciation phenomenon, it favours the presence of the brooding *Platynereis massiliensis* and its settlement rather than the free spawner *P. dumerilii* (Lucey et al. 2015). Although the occurrence of the

brooding species outside vent systems is not precluded, and *vice versa* for *Platynereis dumerilii*. This selection might be correlated with a higher susceptibility of pelagic larval stages to low pH conditions (Dupont et al. 2008, Kurihara 2008, Byrne 2011). On the other hand, a sort of buffering effect may derive from the microhabitat conditions inside the parental tubes of brooding species and/or from the photosynthetic process and oxygen production carried by algae where brooding polychaetes live. The serpulid calcifying tubeworm *Spirorbis spirorbis* showed better growth rates on the brown alga *Fucus serratus* when the algal substrate was under light conditions and could therefore photosynthesize (Saderne and Wahl 2012). The calcification of the blue mussel *Mytilus edulis* is facilitated even under acidified conditions thanks to the fluctuation of the carbonate system produced by the diurnal photosynthesis/respiration cycles of the brown alga *Fucus vesiculosus* and the sea grass *Zostera marina* (Wahl et al. 2017). Both parental tube and macroalgae buffering effects can minimize the OA impact creating a microhabitat that favours the embryos' survival, hatching and juvenile development.

The environmental gradients, created by the CO_2 venting activity, coupled with geographic isolation among sampling sites and reproductive barriers to dispersal, have created the conditions of both sympatric (*sym*: same or together, *patris*: country) and allopatric (*allo*: different, *patris*: country) speciation in the genus *Platynereis.* This speciation phenomenon have lead to the formation of at least 4 different species, two of which were suspected to belong to *Platynereis dumerilii* and the other two to *P. massiliensis*, grouped as two complexes of siblings that still need to be deeply investigated. Nevertheless, once species identity is resolved, the case of *Platynereis* spp. here discussed could represent a good model to study evolutionary implications of climate change on biodiversity.

Chapter 5

A Next-Generation Sequencing approach (RAD-seq) to unravel *Platynereis dumerilii* and *P. massiliensis* sibling species complexes and their relation with Ocean Acidification

5.1 Introduction

The phylogenetic relationships between closely related taxa represents one of the current challenges that researchers must face. The advent of relatively inexpensive and rapid DNA sequencing techniques has considerably increased the odds in the detection and differentiation of species. Nevertheless, the employment of inappropriate phylogenetic markers (which contain insufficient phylogenetic signals) and the occurrence of conflicts in the gene trees topologies (due to interspecific gene flow or incomplete lineage sorting) (Degnan and Rosenberg 2009) still represent a hindrance in the phylogenetic relationships resolution and species identification. The multilocus data approach, with multiple informative markers, can help in distinguishing between these two processes but, historically, methods to identify a large number of genetic markers were expensive in terms of time and money, especially for non-model organisms (Schlötterer 2004).

Since 2005, the introduction of next-generation sequencing (NGS) technologies has offered the opportunity to shift from gene- to genome-scale, with new chances to identify previously unimaginable numbers of sensitive markers for high-resolution genetic analysis of species. Single nucleotide polymorphisms (as suggested by the acronym SNPs) represent the most common form of genetic variation in both coding and non-coding regions of most animal genomes. Each SNP marker consists of a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position (Vignal et al. 2002). The restriction site associated DNA next-generation sequencing (RAD-seq) is a recent SNPs discovery method which combines enzymatic fragmentation of the genome with high-throughput sequencing, allowing the detection of hundreds or thousands of polymorphic genetic markers across the genome in a single, simple and cost-effective experiment (Baird et al. 2008, Davey and Blaxter 2010). High-molecular-weight genomic DNA from multiple samples is digested with one (or more) restriction enzyme. Digested fragments are ligated to adapters that contain a sample-specific barcode or MID (Molecular Identifier) that end with an overhang matching the restriction enzyme's cut site. Adapter-ligated restriction fragments are sheared and fragments containing restriction site overhangs are amplified, using polymerase chain reaction (PCR) and then sequenced.

The obtained RAD-seq reads can be aligned to reference genome or, whether this it is not available (non-model organisms), reads can be used *de novo* to generate a large set of genetic markers. RAD-seq is also a suitable tool to assess species limits and phylogenetic relationships even in closely related taxa for which traditional DNA sequence approaches have failed to provide well supported solutions. Published RAD-tag sequencing research suggest, in fact, that this approach is promising in cryptic/sibling species delimitation as demonstrated for the eastern Australian sea mullet *Mugil cephalus* (Krück et al. 2013), the deep-sea corals of the genus *Paragorgia* (Herrera and Shank 2016) and the non-model land snail complex *Pyramidula* (Razkin et al. 2016).

The model polychaete *Platynereis dumerilii* (Audouin and Milne-Edwards, 1834) and its sibling *P. massiliensis* (Moquin-Tandon, 1869) are therefore a challenging case for the application of RAD-seq. *Platynereis dumerilii* has become a well-understood Evo-Devo model organism (Tessmar-Raible and Arendt 2003, Simakov et al. 2013, Zantke et al. 2014) and a high-coverage reference genome has been generated, although it is still unpublished, so inaccessible to the scientific community (Simakov et al. 2013). The genome of this species has a size of ~1 Gbp and it is characterized by a relatively high polymorphism rate (Zantke et al. 2014). *Platynereis massiliensis* is the only known sibling species of *P. dumerilii*, which has an identical adult stage, although it differs for the life history traits, reproductive biology and gamete morphology (Hauenschild 1951, Lücht and Pfannenstiel 1989, Schneider et al. 1992). It was proposed as a model species for a basic Evo-Devo study (Helm et al. 2014) and, although a genome size of 0.39 Gbp has been estimated, no high-coverage sequencing has ever been performed on this species (Zantke et al. 2014). Due to their morphological similarities, the two siblings were likely to be confused; *Platynereis massiliensis* has been neglected and it is not even reported in Mediterranean polychaete check-lists and revisions (Arvanitidis 2000, Viéitez et al. 2004, Castelli et al. 2008, Çinar et al. 2014, Mikac 2015), or in benthic faunal surveys (Valvassori et al. 2015). Recent studies based on the mitochondrial marker cytochrome *c* oxidase subunit I (COI) amplification and reproductive biology observations of the genus *Platynereis*, hypothesized a correlation between the occurrence of the sibling *P. massiliensis* and low pH conditions, typical of the natural acidified CO_2 vent systems (Calosi et al. 2013b, Lucey et al. 2015, Wäge et al. 2017). The low pH conditions of the CO_2 vent systems, considered as natural laboratories to

study the effects of ocean acidification, may generate a selective pressure that could favour brooding species (Lucey et al. 2015, Wäge et al. 2017). Furthermore, it has been demonstrated that both *Platynereis dumerilii* and *P. massiliensis* might represent two different complexes of siblings characterized by high levels of polymorphisms (Wäge et al. 2017).

In this pilot study a NGS technique, the restriction site associated DNA sequencing (RAD-seq), has been employed for the first time to deeply investigate the phylogenetic relationships and resolve population structure of the model species *Platynereis dumerilii* and *P. massiliensis*. The specimens were collected in sampling sites characterized by different pH conditions (acidified *vs* normal pH) along the Italian coasts. The RAD-seq approach aimed to: (i) clarify which phylogenetic relationships subsist between the two *Platynereis* species and resolve their population structure; (ii) evaluate whether each of them can actually represent a complex of sibling species; (iii) verify whether the low pH conditions might affect their genome by comparing population living under acidified conditions *vs* populations living under normal pH conditions. It is hypothesized to obatin results that confirm the occurrence of two different complexes of sibling species, composed by at least two species each, without signs of interspeific gene flow. It is also assumed that species boundaries are influenced, not by the different geographic distribution, but by the native pH conditions of the sampling sites, which is translated into a genomic adaptation.

5.2 Materials and methods

5.2.1 Sample collection

Platynereis spp. specimens were collected from five geographical areas (eight sampling sites) located along the Italian coasts and characterized by normal (Blue Bay – La Spezia, Sant'Anna – Ischia Island, Ponente Bay – Vulcano Island, Santa Caterina – Lecce) and low pH conditions (Castello Aragonese – Ischia Island, Levante Bay – Vulcano Island, Ditella beach Hot/Cold points and Bottaro crater – Panarea Island). Fourteen individuals were sampled in each site, with the exception of Panarea Island with 5 specimens from Hot/Cold points and 9 specimens from Bottaro crater; for a total of 98 individuals analysed. *Platynereis* specimens, identified by the typical sinuous swimming movement, were collected from macroalgae detached by hand (SCUBA diving or snorkelling) in shallow vegetated rocky reef habitats (0.5-2.5 m depth) and then fixed in 95% ethanol and stored at +4 °C until DNA extraction.

5.2.2 DNA extraction and RAD-seq library preparation

Genomic DNA was extracted from each individual using the MagAttract® HMW DNA QIAGEN kit following the manufacturer's instructions for 'Manual Purification of High-Molecular-Weight Genomic DNA from Fresh or Frozen Tissue'. Extracted DNA concentration was measured with the Qubit dsDNA HS Assay kit designed for the Qubit Fluorimeter (Life Technologies) and its integrity was assessed by electrophoresis, migrating about 100 ng DNA on 1% agarose gel. Two restriction-site-associated DNA libraries of 50 samples each (7 specimens from each sampling site and 1 individual belonging to the species *Nereis zonata* as outgroup) were prepared following the methods described by Etter et al. (2011). 500 ng of DNA sample, collected from each individual of the 50 samples batch, were digested in an enzymatic restriction step of 60 min at 37 °C with the *Sbf*I-HF restriction enzyme (NEB). 50 sample-specific customized P1 adapters (compatible for Illumina Sequencing) were then ligated to the digested DNA samples (Fig. 5.1). Each P1 adapter is a double-stranded oligonucleotide that contains a barcode, a short unique sequence of 5bp called MID (molecular

identifier), which allows to track and identify each sample once they are polled together and sequenced in the same reaction. The barcode occurs at the end of the adaptor and is sequenced immediately before sequencing of the DNA fragment, and thus the barcode sequence will appear at the beginning of the sequence reads. After the heat-inactivation of the DNA Ligase, the 50 DNA samples were pooled together in an equimolar ratio and were afterwards sheared to an optimal size of 400 bp with the Covaris S2 system using Covaris microTUBEs (cat# 520045) in a volume of 130 µl for shearing. A Bioanalyzer system with High Sensitivity DNA kit (Agilent Technologies) was used to check the final fragments size. After fragmentation, the volume was reduced to 56 µL using a SpeedVac concentrator, and samples were subjected to standard Illumina library preparation using the NEBNext Ultra DNA Library Prep Kit for Illumina according to manufacturer's instruction with the customized P2 adapter (compatible for Illumina Sequencing) ligated to sample. 16 PCR cycles (10 s 98°C, 75 s 65 °C) were run and a final purification step was performed using AMPure XP beads (Beckman Coulter). The Qubit dsDNA HS Assay kit designed for the Qubit Fluorimeter (Life Technologies) was used to verify the DNA amplification. Each pool containing 50 barcoded samples was sequenced in a single paired-end lane on the Illumina HiSeq 2000 platform. Each step for DNA extraction and RAD-seq library preparation was performed at the EMBL Genomics Core Facility of Heidelberg (European Molecular Biology Laboratory, Heidelberg, Germany). See Appendix 2 for the detailed RAD-seq protocol (Fig. 5.1 as an overview of the RAD-seq protocol).

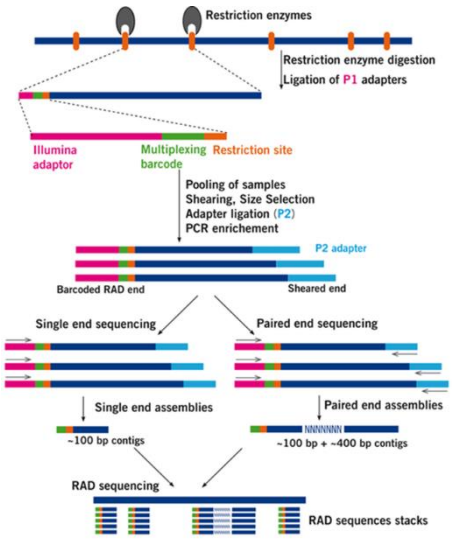

Figure 5.1 Overview of the RAD-seq protocol: restriction enzyme disgestion (one enzyme), adapters ligation (P1), pooling of samples, sharing, size selection, adapter ligation (P2), PCR enrichment, sequencing (from http://www.floragenex.com/rad-seq/, modified).

5.2.3 RAD-tag data analysis

Sequences RAD tags (sequences downstream of restriction sites) were processed with *Stacks* v 1.40 (Catchen et al. 2013). Quality filtering was performed with the 'process_radtags' script with default settings plus barcode distance of 1 to allow barcode rescue (option --barcode_dist). As no reference genome is available for both *Platynereis dumerilii* and *P. massiliensis* (Zantke et al. 2014), short sequence reads were assembled using the *de novo* approach with the 'denovo_map.pl' script. This script run *Ustacks*, *Cstacks* and *Sstacks*. The individual reads were assembled into loci by *UStacks*, setting the number of mismatches between loci when processing single individual to 2 (option -M), the minimum number of identical raw reads to create a stack to 3 (option -m), the SNP model option '--alpha' value of 0.1 was chosen. The number of mismatches allowed during the catalogue creation was set to 2 (option -n, *Cstacks*). After the 'denovo_map.pl' program, the 'populations' script was run to compute population statistics with a minimum number of populations to contain a locus set to 3 (option -p). SNPs with a minor allele frequency (MAF) smaller than 0.05 (option --min_maf) and a genotyping rate smaller than 0.01 were excluded for further analyses. Output that was generated from 'populations' included VCF file, Phylip file and GenePop file. Although paired end sequencing was performed, *Stacks* suite does not employ the pair-end information (as of the time when this is written) so only the first read from each pair was used.

5.2.4 Phylogenetic and population analyses

RAxML v 8.0.9 (Stamatakis 2006, Stamatakis et al. 2008) was used on the CIPRES Portal (Miller et al. 2010) to infer phylogenetic relationships between *Platynereis* spp. specimens using 1,000 bootstrap replicates and NZ1 (*Nereis zonata*) as outgroup. Two datasets were built by separating genotypes of individuals in two species. An R script population specific filter was applied to remove loci with more than 20% of missing genotypes and species monomorphic loci. Each locus was assessed for deviations from Hardy-Weinberg expectations (HWE) at $p < 0.05$ as implemented in Genepop v 4.0 (Rousset 2008), and Linkage disequilibrium (LD) was tested with the Genetics package

in R (Warnes et al. 2012). The sequential Bonferroni method (Rice 1989) for multiple comparisons was applied to correct the significance level. Each genotype dataset was exported to *Structure*, *Genepop* and *Arlequin* software formats using both GenAlEx v 6.5 (Peakall and Smouse 2012) and PGDSpider v 2 (Lischer and Excoffier 2012). Two matrices of Reynolds weighted genetic distances (Drw) (Reynolds et al. 1983) between samples were calculated from their allele frequencies. Neighbor-Joining (NJ) trees were built from these matrices with topological confidences evaluated with 1,000 bootstrap replicates using *Poppr* R package (Kamvar et al. 2014). Each genotype dataset was analysed with the Bayesian clustering approach implemented in *Structure* (Pritchard et al. 2000). For each value of K (number of potential ancestral populations, which ranged from 1 to 5) the genetic ancestry of each individual was estimated based on the admixture model without any *a priori* population assignment; estimations were obtained from 5 independent runs performed for each K using an MCMC of 200,000 interactions after a burn-in of 50,000 interactions. Best K was identified according to the Evanno method (Evanno et al. 2005) as implemented in *StructureHarvester* (Earl and von Holdt 2012). Principal component analysis (PCoA) was performed with the R package *adegenet* (Jombart and Ahmed 2011) without any *a priori* population definition. The outliers loci for *P. massiliensis* dataset were detected with the programs BayeScan v 2.1 (Foll 2012). We calculated q-values as posterior probabilities to estimate the differences in number of putative neutral and selected loci, performing 20 pilot runs with 5,000 iterations and 500,000 iterations MCMC with an additional 50,000 iterations as burn-in. Hierarchical analysis of molecular variance values (AMOVA; 10,000 permutations to test for significance) were assessed for genetic differentiation by the program Arlequin v 3.5 (Excoffier and Lischer 2010). The AMOVA were carried out applying a hierarchical level comparing sampling sites and groups of normal/low pH areas. Pairwise F_{ST} was calculated between populations in Arlequin v 3.5 (Excoffier and Lischer 2010), and *p*-values were computed using a permutation approach (2,000 iterations).

5.3 Results

A total of 477,587 polymorphic loci are detected and a high number of loci are represented by species-specific positions, present in a species but missing in the other. The RAxML dendrogram shows two distinctive clusters identified as *Platyneris* spp. 1 and *Platynereis* spp. 2 (Fig. 5.2). *Platynereis* spp. 1 is supported by a bootstrap value of 55 and branches out in two subclades. The smallest subclade includes 12 samples from Castello Aragonese and 5 samples from Santa Caterina, while the bigger one includes 14 specimens from Levante Bay (VV), 10 from Sant'Anna, 8 from Panarea, 4 from Ponente Bay, 1 individual from Castello Aragonese and 1 from Santa Caterina (Fig. 5.2). *Platynereis* spp. 2 is supported by a bootstrap value of 42 and it includes: 14 individuals from Blue Bay (La Spezia, SP), 10 samples from Ponente Bay (CV), 8 individuals from both Santa Caterina (SC) and Panarea (BO and PA), 4 specimens from Sant'Anna (SA) and 1 individual from Castello Aragonese (S3) (Fig. 5.2).

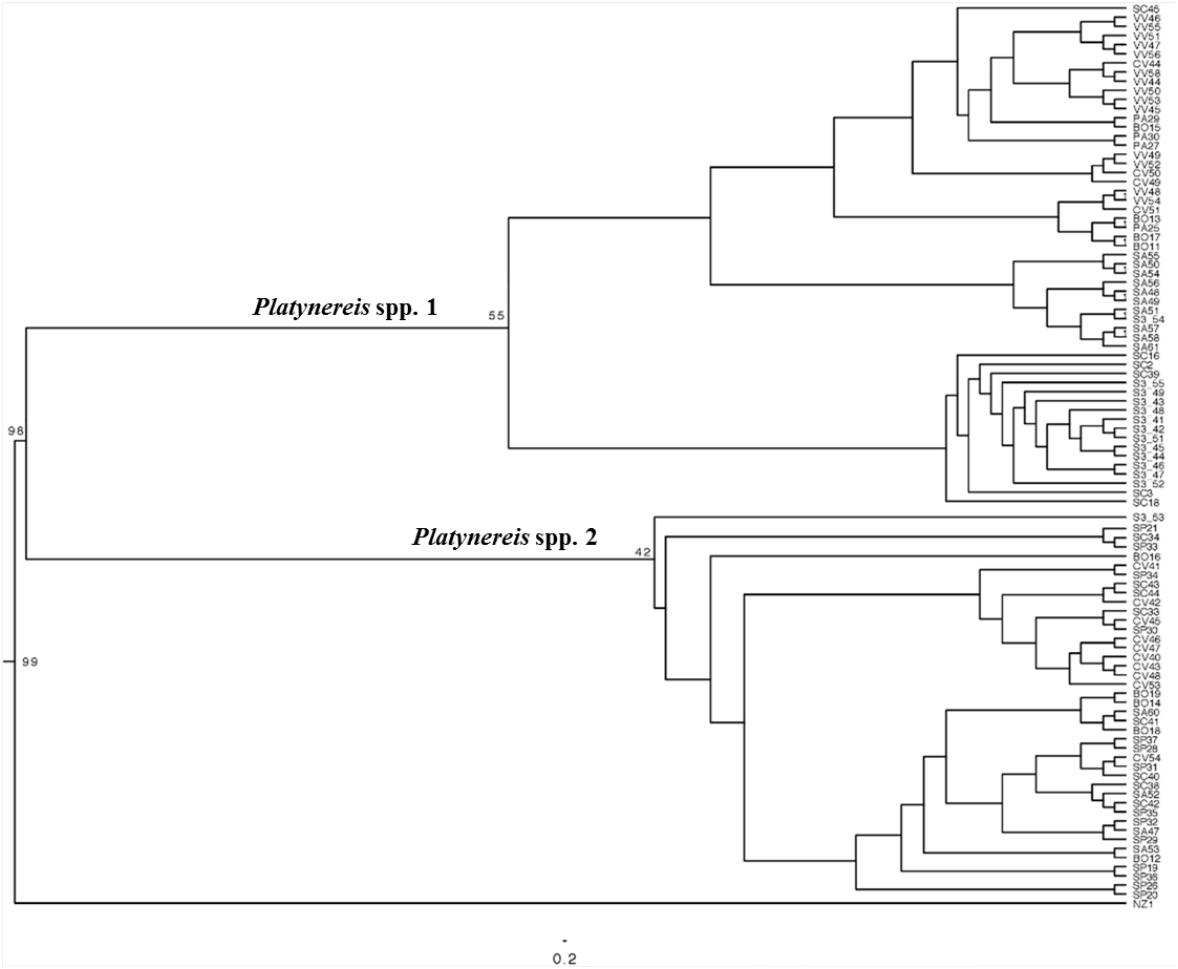

Figure 5.2 RAxML *Platynereis* spp. dendrogram with species 1(bootstrap value 55) and species 2 (bootstrap value 42) branches.

No mixed genotypes and no hybrids between the two species are detected. Two *Platynereis* spp. 1 samples, SC16 and SC45, have been deleted by the successive analyses due to the too high percentages of missing data (99.85% and 99.94% of missing data, respectively). Two datasets (without loci with more than 20% of missing genotypes) are built using 53 individuals for *Platynereis* spp. 1 (2652 SNPs) and 42 individuals for *Platynereis* spp. 2 (4099 SNPs). No LD and HWE departures after sequential Bonferroni correction are detected. New datasets of 2089 SNPs for *Platynereis* spp. 1 and 3262 SNPs for *Platynereis* spp. 2 have been obtained by removing monomorphic loci (563 for species 1; 837 for species 2).

5.3.1 Platynereis *spp. 1 results*

For *Platynereis* spp. 1, the Neighbour-Joining phylogenetic tree shows different clusters (Fig. 5.3). Interestingly, there is a cluster (52 bootstrap) which comprises two subgroups with a clear geographical support: one group with samples from Sant'Anna (SA; 100 bootstrap), plus one individual from Castello Aragonese (S3_54); a second group with samples from Castello Aragonese (S3; 100 bootstrap) and four individuals from Santa Caterina (SC; 100 bootstrap) (Fig. 5.3).

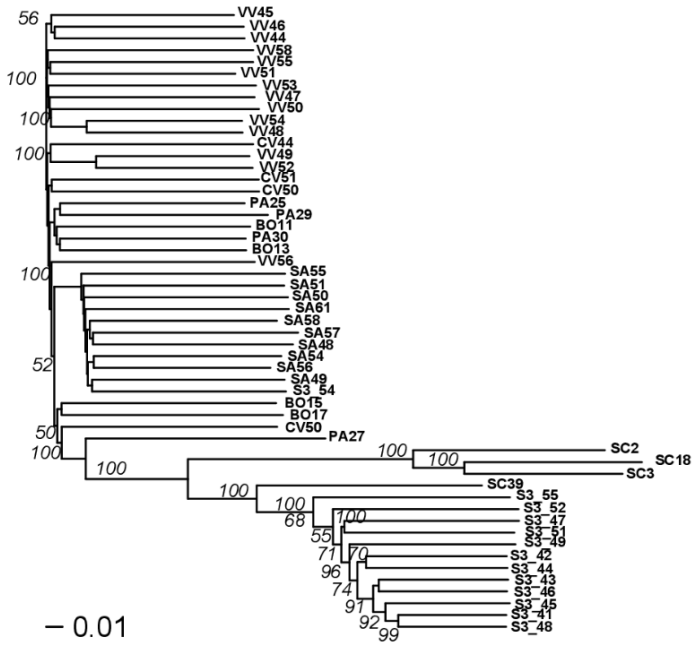

Figure 5.3 *Platynereis* spp. 1 Neighbour-Joining phylogenetic tree.

The Bayesian approach implemented in *Structure* (set K = 2; DeltaK = 14487.02) also shows two clusters mainly consisting of (a) samples from Castello Aragonese (Ischia) and Santa Caterina (Lecce), and of (b) samples from the other areas (Fig. 5.4). Only one individual from Ischia Castello shows a genotype with different clusterization (Fig. 5.4).

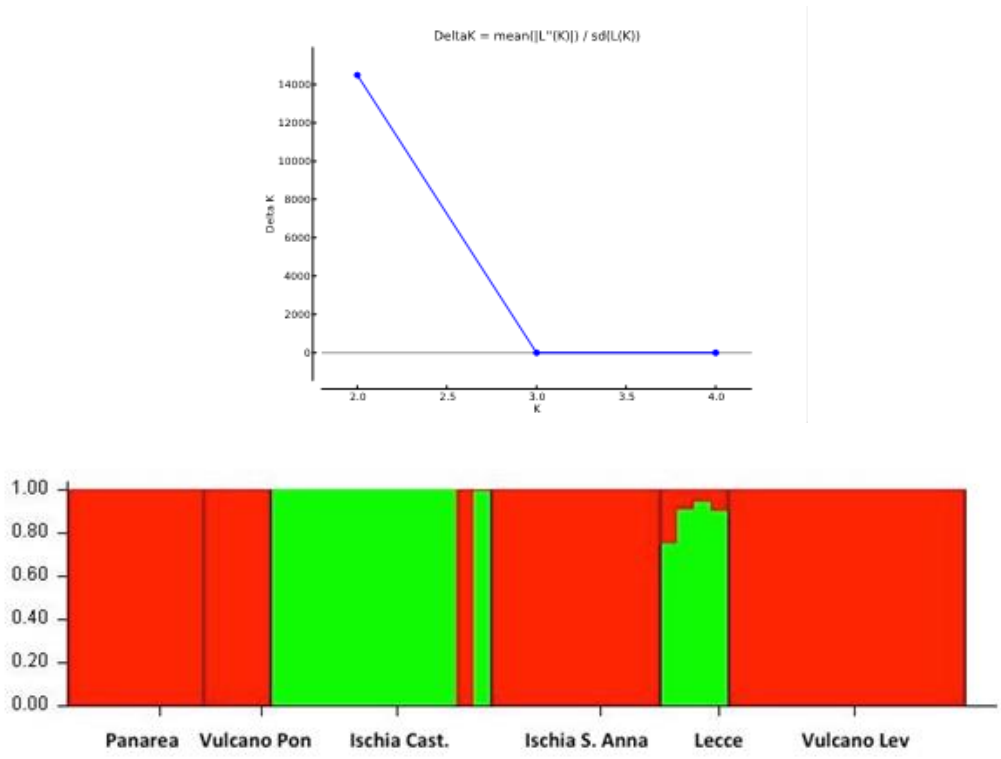

Figure 5.4 *Platynereis* spp. 1 Bayesian approach implemented in *Structure.*

PCoA analysis on the individuals pairwise mean genetic distance, highlights groups differentiation (Fig. 5.5a): a group of 12 samples from Castello Aragonese (Ischia) as the most distinct one along the first axis, which explains 62.00% of the total variance; three samples from Santa Caterina (SC), which explain 30.32% of variance in the second axis, and a group with the other sampling sites (Fig. 5.5a). A second PCoA (mean genetic distance) was performed by removing from the analysis the individuals from Santa Caterina (Fig. 5.5b). The first PCoA axis explains 85.58% of the total genotypic variance and separates individuals of Castello Aragonese vent population from those of Sant'Anna (10 samples plus one from the Castello Aragonese S3_54) and the other geographical areas (8 samples from Panarea, 14 from Levante Bay and 4 from Ponente Bay) (Fig. 5.5b). The second axis of the PCoA explains 2.54% of the total genotypic variance.

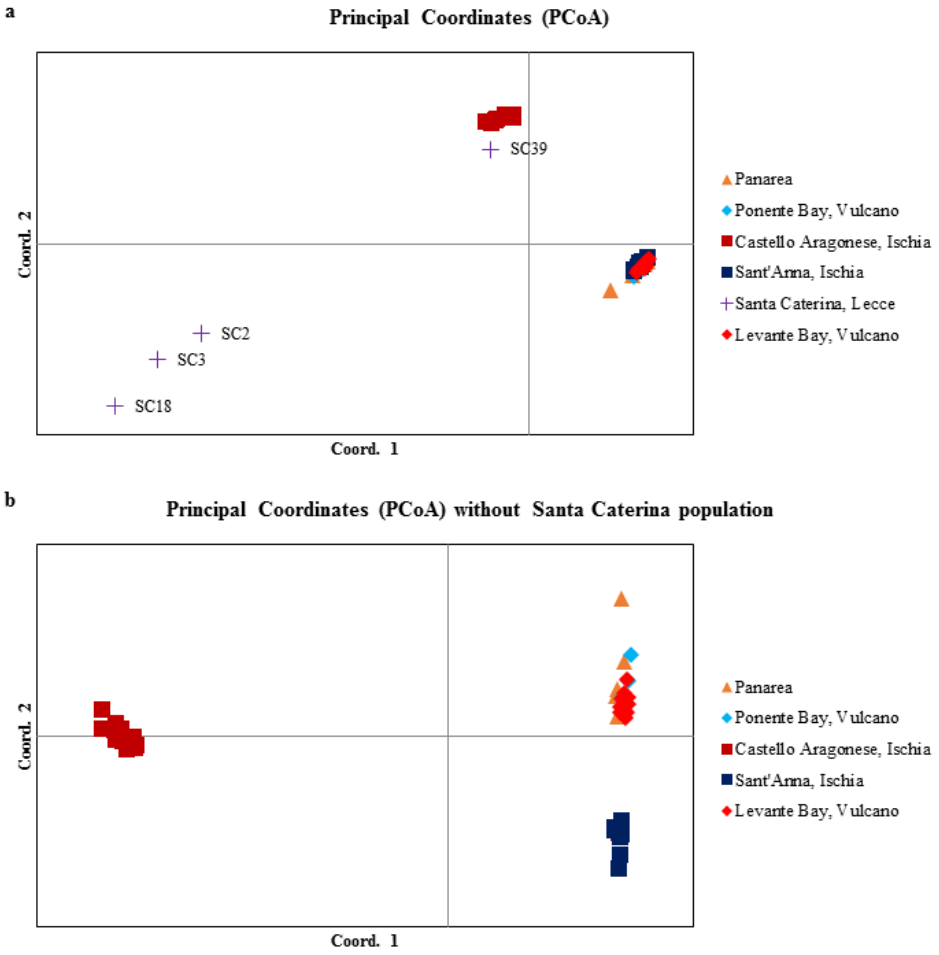

Figure 5.5 Principal Component Analysis (PCoA) via distance matrix with data standardization of *Platynereis* spp. 1 samples. (a) all 53 samples; (b) without Santa Caterina (Lecce) population.

For species 1, the outliers detection with different number of populations was performed: six outliers were detected with six populations based on the different sampling sites (Castello Aragonese and Sant'Anna – Ischia, Levante Bay and Ponente Bay – Vulcano, Panarea, Santa Caterina – Lecce); two outliers were detected with five populations (without Santa Caterina) (Fig. 5.6). The output of the PCoA is the same, indicating that these genes are not responsible for the differentiation.

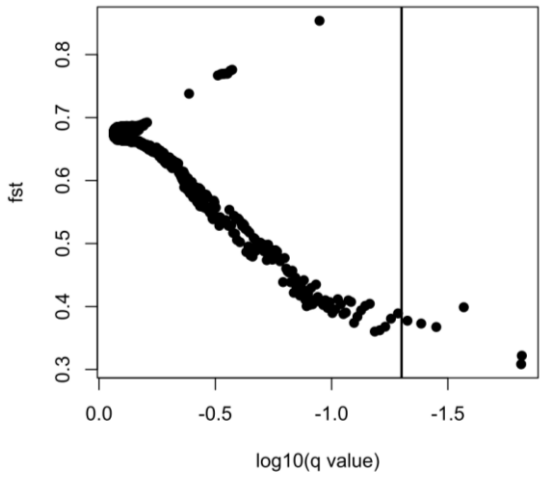

Figure 5.6 Rplot of the six outliers detected considering six populations/sampling sites.

For the AMOVA analysis, groups of samples from areas with different environmental conditions (pH) were compared and non-significant FCT values were obtained (Tab. 5.1). Among all populations (sampling sites), the obtained data show genetic differences. Significant values of variance among populations (FSC = 0.732; p = 0.000) and among individuals within populations (FIS = 0.215; p = 0.021), resulting in the total variation observed among individuals from the whole population (FIT = 0.737; p = 0.000), are shown in Table 5.1. The results of AMOVA indicate that 91.46% of the total variation is attributable to the differences among populations, while only 7.2% is due to the variation among individuals within populations (Tab. 5.1).

Table 5.1 *Platynereis* spp. 1 AMOVA output considering 'groups' of control *vs* acidified areas and 'populations' from different sampling sites. FCT: the variance among groups relative to the total variance; FSC: the variance among subpopulations within groups; FIS: inbreeding coefficient; FIT: overall fixation index.

Source of variation	d.f.	Sum of squares	Variance components	°	Percentage of variation
Among groups	1	166.498	-8.30794	Va	-24.98
Among populations within groups	4	2087.088	30.42421	Vb	91.46
Among individuals within populations	47	636.508	2.39401	Vc	7.2
Among individuals	53	464	8.75472	Vd	26.32
Total	*105*	*3354.094*	*33.26499*		
Fixation Indices					
FCT		-0.24975	p = 0.908	Va	
FSC		0.73183	p = 0.000	Vb	
FIS		0.21473	p = 0.021	Vc	
FIT		0.73682	p = 0.000	Vd	

Pairwise F_{ST} between *Platynereis* spp. 1 populations (sampling sites) highlights statistically significant differentiation levels as reported in Table 5.2. All populations are isolated from each other with the exception of Levante Bay – Panarea and Levante Bay – Ponente Bay.

Table 5.2 Pairwise F_{ST} values between *Platynereis* spp. 1 populations (sampling sites) with respective *p* values in brackets; the boxes highlighted in gray correspond to statistical significant values ($p < 0.05$).

	Panarea	**Ponente**	**Castello**	**SAnna**	**SCaterina**	**Levante**
Panarea	0					
Ponente	0.08901 (0.017)	0				
Castello	0.78692 (0.000)	0.77136 (0.000)	0			
SAnna	0.37489 (0.000)	0.36939 (0.000)	0.79102 (0.000)	0		
SCaterina	0.83338 (0.001)	0.86056 (0.021)	0.30254 (0.029)	0.82281 (0.001)	0	
Levante	0.02602 (0.084)	0.03497 (0.119)	0.79195 (0.000)	0.31089 (0.000)	0.79608 (0.000)	0

5.3.2 Platynereis *spp. 2 results*

For *Platynereis* spp. 2, the Neighbour-Joining phylogenetic tree shows a subcluster supported by a high value of bootstrap (100), which includes all samples from Ponente Bay (CV), three samples from Santa Caterina (SC), and two samples from Blue Bay (La Spezia, SP) (Fig. 5.7). The other samples do not show a clear geographical pattern and the same cluster includes few specimens from the acidified areas of Panarea (5 samples from Bottaro crater, BO) and Castello Aragonese (S3_53) (Fig. 5.7).

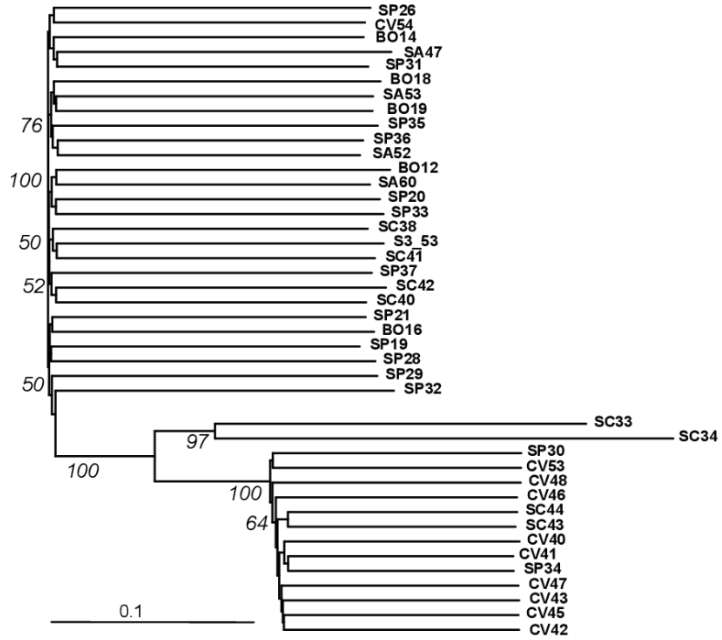

Figure 5.7 *Platynereis* spp. 2 Neighbour-Joining phylogenetic tree with bootstrap values.

The Bayesian approach implemented in *Structure* shows two main clusters (set K = 2; DeltaK = 4760.29) (Fig. 5.8). The distribution of genotypes is structured among populations (sampling sites) without clear geographical pattern and most sampling areas still appear undifferentiated (Fig. 5.8).

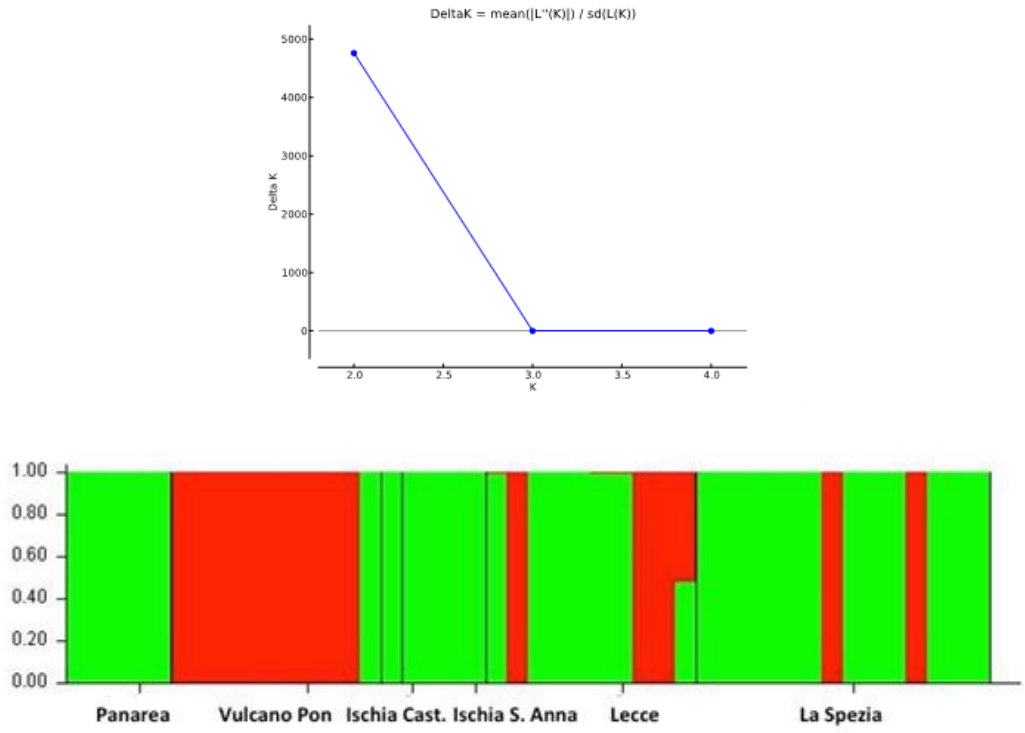

Figure 5.8 *Platynereis* spp. 2 Bayesian approach implemented in *Structure*.

The PCoA analysis of the individuals pairwise mean genetic distance, highlights a differentiation in two main groups, explained by 41.58% of the total variance in the first axis, and also samples from Santa Caterina (SC33 and SC34), which resulted by 35.60% of the total variance in the second axis (Fig. 5.9a). The first group includes 9 individuals from Ponente Bay, 2 from Santa Caterina and 2 from Blue Bay. The second cluster includes 12 samples from Blue Bay, 5 from Panarea, 4 from Santa Caterina, 4 from Sant'Anna, 1 from Ponente Bay and 1 from Castello Aragonese. A second PCoA (mean genetic distance) was performed without considering Santa Caterina individuals (Fig. 5.9b). The first PCoA axis explains 62.66% of the total genotypic variance and separates individuals in two main groups, as in the previous analysis (Fig. 5.9b). The second axis of the PCoA explains 2.74% of the total genotypic variance.

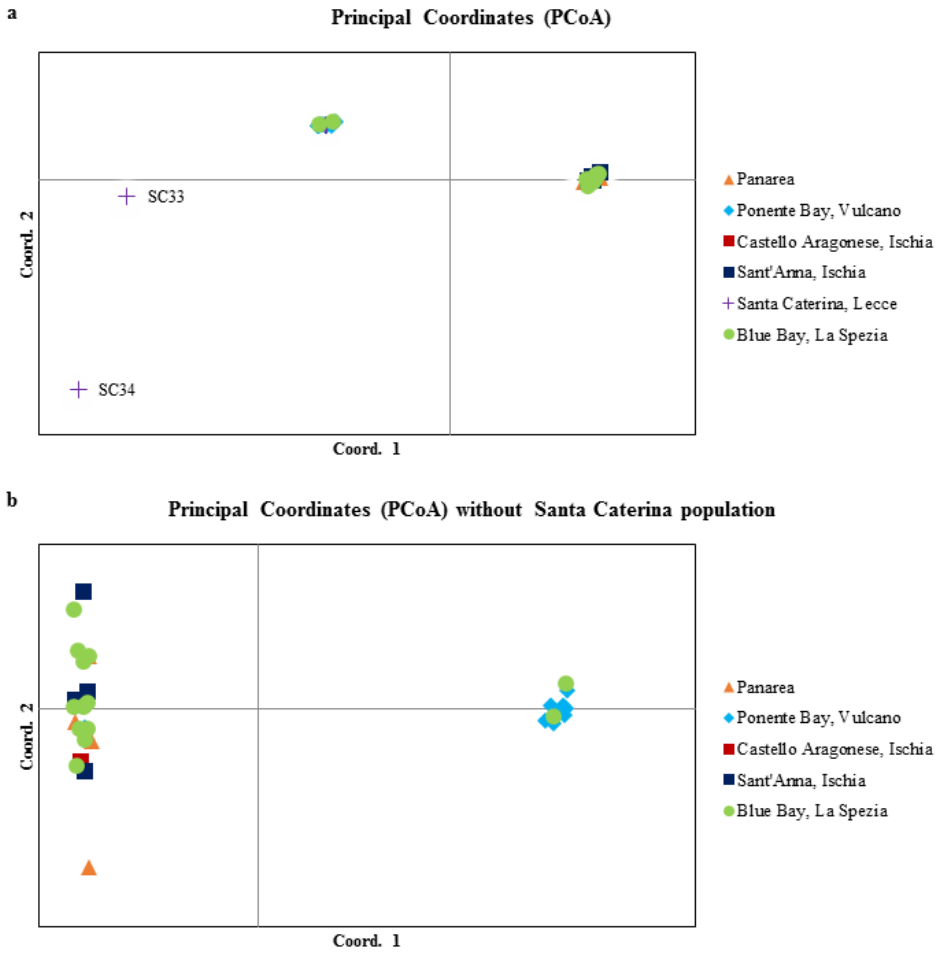

Figure 5.9 Principal Component Analysis (PCoA) via distance matrix with data standardization of *Platynereis* spp. 2 samples. (a) all 40 samples; (b) without Santa Caterina (Lecce) population.

The results of AMOVA indicate that 36.73% of the total variation is attributable to the differences among populations (sampling sites) and 32.57% is due to the variation among individuals within populations (Tab. 5.3). In fact, significant values of variance are shown among populations (FSC = 0.371; p = 0.000) and among individuals within populations (FIS = 0.524; p = 0.000), resulting in the total variation observed among individuals from the whole population (FIT = 0.704; p = 0.000) (Tab. 5.3). No significant values of variance are detected among acidified and normal pH groups (FCT = 0.011; p = 0.533) (Tab. 5.3).

Table 5.3 *Platynereis* spp. 2 AMOVA output considering 'groups' of control *vs* acidified areas and 'populations' from different sampling sites. FCT: the variance among groups relative to the total variance; FSC: the variance among subpopulations within groups; FIS: inbreeding coefficient; FIT: overall fixation index.

Source of variation	d.f.	Sum of squares	Variance components	°	Percentage of variation
Among groups	1	60.380	0.13689	Va	1.14
Among populations within groups	4	300.360	4.41948	Vb	36.73
Among individuals within populations	38	433.021	3.9124	Vc	32.57
Among individuals	44	156.500	3.55682	Vd	29.56
Total	*87*	*950.261*	*12.03242*		
Fixation Indices					
FCT		0.01138	$p = 0.533$	Va	
FSC		0.37152	$p = 0.000$	Vb	
FIS		0.52424	$p = 0.000$	Vc	
FIT		0.7044	$p = 0.000$	Vd	

Pairwise F_{ST} between *Platynereis* spp. 2 populations (sampling sites) highlights statistically significant differentiation as reported in Table 5.4. Ponente Bay population is isolated by Panarea, Sant'Anna, Santa Caterina and La Spezia; as well as Santa Caterina from both Panarea and Sant'Anna.

Table 5.4 Pairwise F_{ST} values between *Platynereis* spp. 2 populations (sampling sites) with respective *p* values; the boxes highlighted in gray correspond to statistically significant values ($p < 0.05$).

	Panarea	Ponente	Castello	SAnna	SCaterina	LaSpezia
Panarea	0					
Ponente	0.66199 (0.001)	0				
Castello	0.0398 (0.999)	0.06772 (0.992)	0			
SAnna	0.02665 (0.178)	0.66725 (0.000)	0.05696 (0.433)	0		
SCaterina	0.21691 (0.048)	0.30315 (0.025)	0.17323 (0.440)	0.21679 (0.047)	0	
LaSpezia	0.02967 (0.101)	0.51512 (0.000)	-0.02241 (0.392)	0.02355 (0.436)	0.08685 (0.137)	0

5.4 Discussion

The advent of the next-generation DNA sequencing (NGS) tools represents a new opportunity for gathering genome-scale sequence data; it is transforming the way in which biologists generate data to study natural populations. An efficient, flexible and inexpensive genotyping approach that allows the simultaneous discovery of tens of thousands of genetic markers, is the restriction site associated DNA sequencing (RAD-seq). This technique offers a reduced representation of the genome by sampling those regions near restriction enzyme cut sites, with a great potential of applications to the population genomics of species without a reference genome and species without clear delimitation boundaries, such as sibling/cryptic species. The marine invertebrate polychaete species *Platynereis dumerilii* represents a peculiar case of an Evo-Devo model species for which no reference genome is available (Simakov et al. 2013). Based on the recent reappraisal of the only known sibling species *Platynereis massiliensis* (Valvassori et al. 2015), and on the discovery of the putative existence of complexes of cryptic species for both *P. dumerilii* and *P. massiliensis*, whose occurrence seems to be correlated with the pH conditions (Lucey et al. 2015, Wäge et al. 2017), a population genomics approach was desirable to resolve the phylogenetic relationship between *Platynereis* sibling species.

The use of a NGS approach to study the challenging case of *Platynereis* have lead to several interesting insights, first of all validating the occurrence of two different complex of sibling species. A comparison with the phylogenetic tree obtained by the COI amplification of the same samples (Chapter 4), proves that the two identified species are *Platynereis dumerilii* and *P. massiliensis*. *Platynereis* spp. 1 corresponds to the *P. massiliensis* sibling complex with clade 1 that includes 12 samples from Castello Aragonese (S3) and 5 samples from Santa Caterina; while clade 2 includes the remaining from Levante Bay (VV), Sant'Anna, Panarea, Ponente Bay, Castello Aragonese and Santa Caterina (Fig. 5.10). On the other hand, *Platynereis* spp. 2 coincides with the *P. dumerilii* sibling complex. Clade 3 corresponds to the group with 9 specimens from Ponente Bay (CV), 3 from Santa Caterina and 2 from La Spezia. Clade 4 corresponds to the remaining specimens from Blue Bay (SP), Ponente Bay, Santa Caterina, Panarea (BO and PA), Sant'Anna and Castello Aragonese, although they are not all grouped together (Fig. 5.10).

As already showed by the COI amplification, Levante Bay is a site solely dominated by specimens of clade 2 (*Platynereis massiliensis* complex) while, Blue Bay by specimens belonging to the *P. dumerilii* complex (clade 3 and 4).

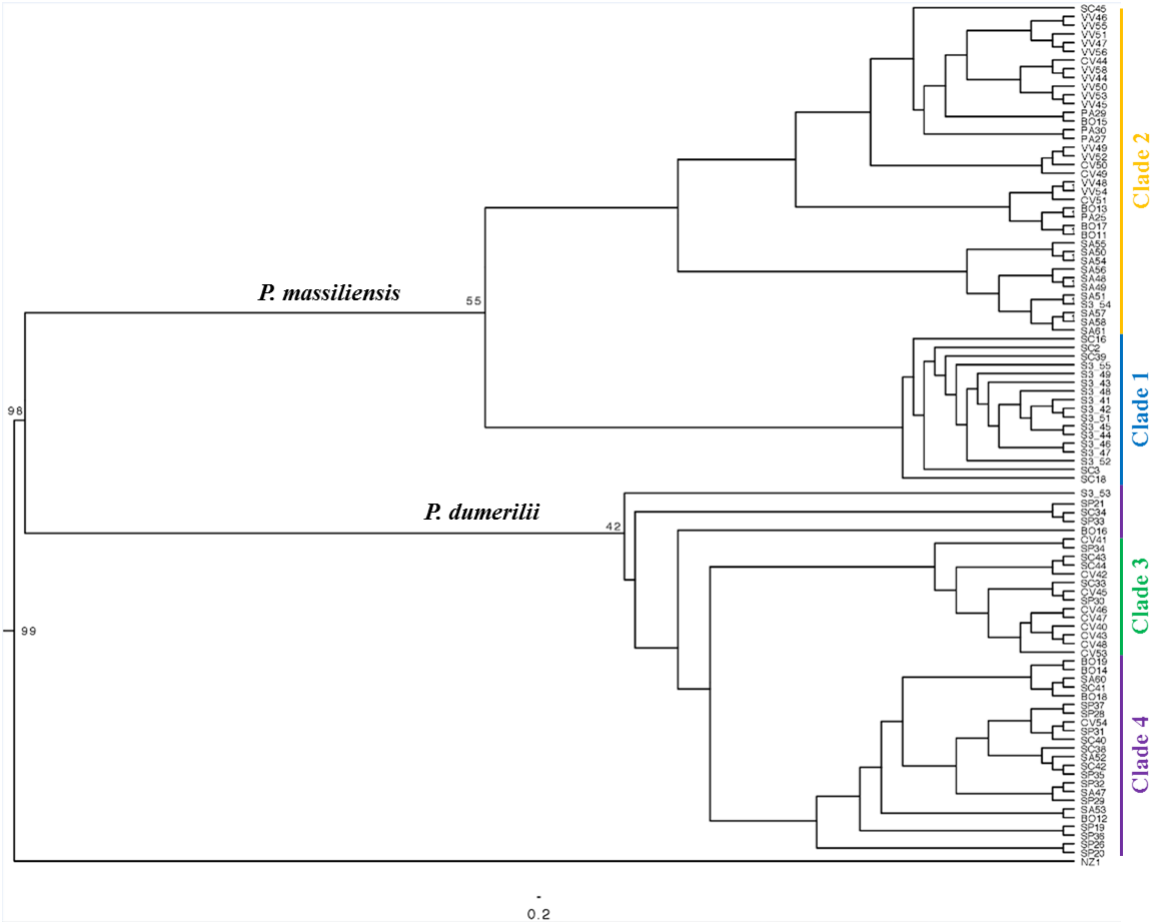

Figure 5.10 RAxML *Platynereis* spp. dendrogram with *P. massiliensis* (bootstrap value 55) and *P. dumerilii* (bootstrap value 42) branches and colorful sidebars based on the COI clade at which specimens belong (clade 1 - blue, clade 2 - yellow, clade 3 - green, clade 4 - purple).

The results clearly show the occurrence of different significant clusters in *Platynereis massiliensis*. This species is subdivided into at least two different clades, one of them includes specimens from Castello Aragonese (S3) and Santa Caterina (Lecce). The PCoA demonstrates that no gene flow occurs among the main groups. It seems that no geographical coherence occurs in the clusters' composition, however, the removal of the Santa Caterina population from the analysis highlights high levels of differentiation (85.58%) between the nearby populations of Castello Aragonese and Sant'Anna (approximately 600 metres from each other). A high connectivity would be expected among these close populations, exhibiting relativity low levels of genetic structure. The outliers detection results do not explain any adaptive process to different

pH conditions; therefore the high differentiation levels might be caused by the occurrence of two different siblings of *Platynereis massiliensis* (as shown by Bayesian approach results, Fig. 5.4) which co-occur in Ischia Island. Cryptic polychaetes are not restricted to widely distributed nominal species, but are often found in sympatry, in the same habitat or in very close vicinity (Blank et al. 2006, Paxton 1979, Kikuchi and Yasuda 2006, Nygren et al. 2010). Another possible explanation might be the 'founder effect' caused by a limited number of colonists that settled at the Castello Aragonese vent area with consequent isolation caused by the peculiar pH conditions. The differentiation process might also be facilitated by the brooding nature of *Platynereis massiliensis*, whose reproductive cycle is not characterized by the occurrence of planktonic larval stages, as *P. dumerilii*, but by embryos that undergo a semi-direct development into juvenile worms inside the parental tube with consequent limited dispersal capabilities (Schneider et al. 1992). The considerable genetic differentiation from nearby sampling sites might be due to the reduced dispersal rates that lead to inbreeding within populations that are not stable over many generations, as in the case of the brooder spirorbid polychaete species *Pileolaria pseudomilitaris* (Beckwitt 1980). Few individuals of the *Platynereis massiliensis* complex from Santa Caterina form a small subclade, which is genetically differentiated. This is likely due to the accumulation of mutations caused by different evolutionary processes, which occurred because of the elevated geographical distance between Tyrrhenian and Ionian populations. The AMOVA results highlight no significant differences in the comparison between *Platynereis massiliensis* from the acidified sites *vs* normal pH sites. On the other hand, a significant variability is found among populations and among individuals within populations. A high differentiation among individuals was expected due to the elevated number of markers obtained with the RAD-seq technique. The pairwise F_{ST} analysis highlights a high level of genetic differentiation with the sole exception of the panmictic populations of Levante – Ponente Bays and Levante Bay – Panarea, likely due to their geographical proximity (these two islands of the Aeolian Archipelago are approx. 20 miles apart). The high *Platynereis massiliensis* populations' differentiation among sampling sites is likely due to a higher capability of this species to adapt to different habitat conditions coupled with its low dispersal potential.

As regards *Platynereis dumerilii*, two different clades are highlighted with a composition that is not correlated with the geographical distribution and without signs

of gene flow between the two genetic groups. A small subcluster of Santa Caterina individuals is differentiated from the other samples and the accumulation of genetic mutations mediated by the geographical distance between Tyrrhenian and Ionian populations could represents a valid explanation. The Ionian Sea is located in the Eastern Mediterranean and therefore the Santa Caterina population is more isolated compared to the others, also for a species with pelagic larval stages (Mamuris et al. 1998). Significant variability has been found among populations and among individuals within populations while, according to the AMOVA results, no differences have been detected based on the pH conditions of the collection sites. In the pairwise F_{ST} analysis, the Ponente Bay population is the most differentiated one as it is isolated by Panarea, Sant'Anna, Santa Caterina and La Spezia. Low levels of alleles sharing are also observed between Santa Caterina – Panarea and Santa Caterina – Sant'Anna. No significant differences are detected in the populations' comparison such as Castello Aragonese and Sant'Anna; this might be due to the low number of individuals analysed (1 and 3, respectively), while at least five individuals would be necessary to obtain informative results (Vedrami et al. 2017).

The results highlighted by the RAD-seq approach confirm, as previously hypothesized by Wäge et al. (2017) that both *Platynereis dumerilii* and *P. massiliensis* represent two different complexes of sibling species. Both of them are characterized by at least two species each and do not hybridize to each other. In both complexes, the Santa Caterina population is a genetically differentiated group likely due to the occurrence of a geographical isolation that reduces the genetic flow between the populations of the Tyrrhenian and the Ionian Seas (Eastern Mediterranean). Increased environmental distance may result in increased genetic distance as individuals or populations adapt to different environmental factors in different regions (Frankham et al. 2002). A similar phenomenon has already been observed for other Mediterranean marine organisms which show high motility, such as the red mullet fish *Mullus barbatus* (Maggio et al. 2009), or a pelagic life habit, as the dinoflagellate *Alexandrium minutum* (Casabianca et al. 2011). In both *Platynerei*s species complexes here studied, there is no correlation with the original habitat pH conditions, the only differences detected are based on the different sampling sites (although the sites co-vary with pH conditions) and, despite the different theoretical dispersal ability of the early developmental stages, the *P. massiliensis* brooding complex does not show a more

restricted distribution. 66% of the individuals identified as *Platynereis massiliensis* were sampled in low pH areas, while 85.7% of the *P. dumerilii* samples were collected in normal pH zones. Although the correlation is not statistically significant, a sort of preference of *Platynereis massiliensis* for the acidified conditions has been denoted, and for normal pH in *P. dumerilii*. The predominance of *Platynereis massiliensis* in acidified areas has already been observed and explained as a successful brooding habit in stressful conditions (see Chapter 4; Wäge et al. 2017). In order to resolve population structure over a small geographical scale and obtain results with a higher statistical power, it would be desirable to repeat the same RAD-seq approach working on a higher number of *Platynereis* samples per population, at least five (Vendrami et al. 2017).

Chapter 6

Antioxidant capacity comparison between *Platynereis* sibling species according to pH conditions

6.1 Introduction

Marine organisms are continuously exposed to a wide range of environmental factors, varying over both temporal and spatial scales, which may represent a source of oxidative stress that leads to ROS production. The term ROS is an acronym which stands for 'reactive oxygen species': endogenous and highly reactive oxygen (RNS with nitrogen) –bearing molecules. They are commonly produced at low concentration levels during several natural cellular pathways of aerobic metabolism. Under basal and stable conditions, their adverse effects are prevented by antioxidant defence systems consisting of a range of low molecular weight scavengers and enzymes. In stressful conditions, this balance may be broken leading to uncontrolled formation of ROS which translates into cellular oxidative damages against lipids, proteins and DNA, impairing the normal cellular functions. This shift in the oxidant - antioxidant balance in favour of oxidants is termed 'oxidative stress'. The effects of Global Climate Change (GCC) in the marine environment, with increasing temperature, higher absorption of solar radiation and lowering pH (known as ocean acidification, OA), have implications at the level of the antioxidant activity of several marine organisms (Tomanek et al. 2011, Zhang et al. 2011, Matoo et al. 2013, Matozzo et al. 2013, Vehmaa et al. 2013). The continuous increase in carbon dioxide (CO_2) emissions in the atmosphere could lead the current levels of 380 ppm to reach 800 ppm, by 2100 (Feely et al. 2004). When the atmospheric CO_2 diffuses passively into the ocean surface, it causes the alteration of carbonate chemistry with a pH reduction from 0.3 to 0.5 units. This change is predicted to occur by the end of the 21st century (Raven et al. 2005, IPCC 2014). Tomanek et al. (2011) suggested that elevated CO_2 levels might cause oxidative stress by increasing the production of ROS either indirectly, by lowering intra- and extracellular pH, which may enhance the Fenton reaction, or directly, by interacting with other ROS to form more free radicals. Matozzo et al. (2013) found that the exposure to different combinations of pH, temperature and salinity, at levels similar to GCC predictions, can affect the biochemical responses to oxidative stress in two marine bivalves, the mussel *Mytilus galloprovincialis* and the striped Venus clam *Chamelea gallina*, leading to an increase in the antioxidant enzymatic activities.

The exposure of the copepod *Centropages tenuiremis* to elevated pCO_2, was demonstrated to cause significant changes in some antioxidant enzymatic systems (GPx, SOD and GSH) (Zhang et al. 2011). Although the interest in how OA affects the antioxidant capacity of marine organisms is growing, there is still little knowledge about how the antioxidant defence system of the benthic compartment is affected. A realistic and informative approach to investigate the effects of low pH/elevated pCO_2 is represented by the study of natural CO_2 vent systems that are located in different parts of the world (Hall-Spencer et al. 2008, Kroeker et al. 2011, Johnson et al. 2012, Fabricius et al. 2011, 2014, Goffredo et al. 2014). Such natural laboratories have provided important information that allows the identification of putative 'sensitive' and 'tolerant' species to low pH conditions.

Inside the benthic community, polychaetes represent a key group of organisms which appears particularly abundant along the Castello Aragonese CO_2 vent system of Ischia Island (Italy) (Cigliano et al. 2010, Kroeker et al. 2011, Ricevuto et al. 2014), and was therefore studied to evaluate the effects of low pH conditions. The polychaete *Platynereis dumerilii* (Audouin and Milne-Edwards, 1834) was identified by Calosi et al. (2013b) as a tolerant species, and was employed as a model species by Ricevuto et al. (2015a) to evaluate the antioxidant capacity with an *in situ* reciprocal transplant experiment. The results of the total oxyradical scavenging (TOSC) assay after 30 days of reciprocal transplant testing, carried out with animals originating from vents and control areas, and exposed to both acidified/control conditions in experimental areas of Castello Aragonese at Ischia, reveal the absence of antioxidant variations after translocation (from vent conditions to control and *vice versa*). This indicates that elevated pCO_2/low pH has no effect on the antioxidant efficiency. However, a long-term adaptation to a greater prooxidant challenge in the vent site has been confirmed by more elevated basal antioxidant efficiency of the native 'vent population' (Ricevuto et al. 2015a), likely due to the need for greater protection in conditions of chronic oxidative exposure (Regoli et al. 2011). Preliminary genetic results and reproductive biology observations carried out on *Platynereis* spp. specimens, collected from the same acidified and control areas of Ischia, demonstrated that the vent population belonged to *P. massiliensis* (Moquin-Tandon, 1869), the neglected sibling species of *P. dumerilii* (Calosi et al. 2013b, Lucey et al. 2015, Valvassori et al. 2015).

As sibling species, they are morphologically indistinguishable at the sexually immature adult stage but differ in life history traits, reproductive biology and gamete morphology (Haueschild 1951, Schneider et al. 1992, Valvassori et al. 2015) (see Chapter 3). The results of antioxidant assay and genetic/reproductive biology analyses indicate that the two siblings, *Platynereis dumerilii* and *P. massiliensis*, have different antioxidant system capacities.

We investigated the effects of low pH conditions on the antioxidant capacity of the putative *Platynereis massiliensis* (collected in the Castello Aragonese vent system) and *P. dumerilii* (from the control site of Sant'Anna) through: (i) background analyses of individuals collected from natural populations (inside and outside the vent system) to compare basal levels of biological responses to oxidative stress (enzyme activities and Total Oxyradical Scavenging Capacity, TOSC); (ii) a long-term (30 days) laboratory translocation experiment with three different pH treatments (normal, low pH and extreme low pH conditions) to evaluate the biological responses of antioxidant parameters (as single antioxidant and TOSC) to pH levels between the two populations.

6.2 Materials and methods

6.2.1 Study areas, sample collection and processing

The study was conducted in Ischia (Gulf of Naples, Italy), an island of volcanic origin famous for the presence of numerous sub-marine CO_2 vent systems (Tedesco 1996, Gambi 2014), including the Castello Aragonese on the north-eastern side (40° 43.84 N, 13° 57.08 E). The vents, characterized by ambient seawater temperature and occuring between 0.5 and 3.0 m in depth, originate a gradient of seawater acidification on both the north and south sides of the Castello islet. According to Kroeker et al. (2011), three different pH zones can be identified along a rocky reef of approximately 150 m in length on each side of the islet: a control area with normal pH conditions and no venting activity (N1 and S1), an intermediate area with moderate vent activity and low pH conditions (N2 and S2), and a high venting activity area characterized by extreme low pH conditions (N3 and S3). The control site called Sant'Anna rocks, characterized by normal pH, is located in Cartaromana Bay approximately 600 m from the south side of Castello Aragonese (Ricevuto et al. 2015a).

Platynereis spp. samples were collected from macroalgal thalli belonging to the species *Halopteris scoparia*, *Jania rubens* and *Dictyota* spp., at which they are associated to in the south-acidified sites of Castello Aragonese (named as S3 and S2 in previous papers e.g. Cigliano et al. 2010, or as low pH and extreme low pH in Kroeker et al. 2011), and in the normal/control pH area of Sant'Anna. The samplings were carried out in four different periods over two years: April 2016, October 2015-2016 and February 2017. A non-quantitative but standardized sampling was performed collecting three cotton fabric bags (25 x 25 cm, approximately 500 gr. w.w. of macroalgae) full of thalli and transported them to the laboratory inside cool boxes within one hour after collection. Once in the lab, a few marcoalgal thalli at time were shaken into big plastic trays to dislodge the macrofauna and the key *Platynereis* spp. species were identified thanks to the typical sinuous swimming movement, gently picked up with a plastic pipette and stored in Petri dishes. Specimens destined to background analyses (April 2016, October 2016 and February 2017) were immediately transferred into separated 1.5 ml microcentrifuge tubes (approximately 5-10 individuals per eppendorf, according to the body mass of the collected samples) and frozen at -80 °C.

Individuals of the translocation experiment (collected in October 2015) were maintained at the same seawater temperature and photoperiod of the collection time (T = 21 °C, L:D = 12h:12h), in crystallizing dishes (vol. = 200 ml, approximately 20 individuals per dish) supplied with filtered seawater (0.22 μm) from the sampling sites until the setting of the laboratory translocation experiment.

6.2.2 Translocation experiment: set-up and design

The laboratory translocation experiment was performed in the wet laboratories of Villa Dohrn Benthic Ecology Centre of Ischia (Research Unit of the Stazione Zoologica Anton Dohrn, Naples). Tanks filled with seawater were maintained at three different pH conditions: normal $pH_{(NBS)}$ 8.00 ± 0.05, low $pH_{(NBS)}$ 7.70 ± 0.05 and extreme low $pH_{(NBS)}$ 7.40 ± 0.05. The pH values were manipulated through bubbling seawater with CO_2 gas using the 'CO_2 energy professional' kit regulated by digital pH controllers (Ferplast mod. AQ2001) connected to pH electrodes mounted in each incubation tank. The seawater pH value of each tank was also checked three times a day with the portable pH-meter Mettler Toledo mod. SevenGo equipped with the InLab4 13SG electrode. Seawater was pumped constantly inside the tanks through external aquarium filter units (Pratiko Askoll) and approximately ¼ of the total volume was periodically replaced with fresh seawater. The seawater temperature of each tank was measured three times a day and was maintained at 21 ± 0.5 °C using the Teco conditioners mod. Micro. Salinity and dissolved oxygen saturation were maintained at constant levels and checked once a day: the seawater salinity (38 PSU) was measured with the digital refractometer Milwaukee mod. MA877 having an accuracy of ± 1 PSU; dissolved oxygen saturation was checked with an YSI mod. ProODO instrument. Three replicates were considered for each pH treatment with 25 specimens per replicate from Sant'Anna and 30 specimens per replicate from Castello (S2-S3 areas). The worms of each replicate were inserted inside purpose-built cylindrical chambers (white PVC, diam. = 4 cm, length = 11 cm), closed at both ends by a plankton net (mesh size = 100 μm), as in Ricevuto et al. (2015a). Small portions of the macroalga *Dictyota* spp. were added as feeding source and substrate for the worms.

The translocation experiment took place from the 15th of October to the 16th of November 2015, for a total of 30 days period of the worms' exposure to the experimental conditions as reported in Table 6.1.

Table 6.1 Summarizing table of the laboratory experimental set-up; cc: control conditions.

Putative species	Sampling site	Sampling site pH	pH treatment	
P. dumerilii	Sant'Anna	~ 8.08	Normal	8.00±0.05 **cc**
			Low pH	7.70±0.05
			Extreme low pH	7.40±0.05
P. massiliensis	Castello Aragonese	~ 7.70	Normal	8.00±0.05
			Low pH	7.70±0.05 **cc**
			Extreme low pH	7.40±0.05

After 30 days the number of live individuals were recorded and survivors were immediately transferred to 1.5 ml microcentrifuge tubes (eppendorfs) in subsample pools of 5-10 individuals each, labelled and stored at -80 °C for subsequent analyses. Afterwards, samples were sent in dry ice to the Department of Life and Environment Sciences of the Polytechnic University of Marche (UNIVPM) in Ancona (Italy) for the oxidative stress analyses.

6.2.3 Sample preparation and antioxidant analyses

6.2.3.1 Antioxidant enzymes analyses

For the analysis of the antioxidant enzymes, each subsample pool were homogenized (1:10 w:v) in 100 mM of K-phosphate buffer (pH 7.5) containing NaCl (1.5%), 0.1 mg mL^{-1} phenylmethylsulphonyl fluoride (PMSF), 0.1 mg mL^{-1} bacitracin and 0.008 TIU mL^{-1} aprotinin as protease inhibitors (Aprotinine and Bacitracine). After centrifuging at 100,000 x g for 70 min at 4 °C, supernatants were collected and kept at -80 °C until required. All enzymatic measurements were carried out using a Varian (Model Cary 3) spectrophotometer at the constant temperature of 18° C.

Catalase (CAT) is a heme-containing protein that has a detoxifying effect towards the hydrogen peroxide (H_2O_2), catalysing the reaction which leads to the formation of water and oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

CAT was measured by the decrease in absorbance at 240 nm ($\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$) due to H_2O_2 consumption (12 mM H_2O_2) in 100 mM K-phosphate buffer (pH 7.0).

Glutathione S-transferases (GST) are metabolic isoenzymes involved in detoxification of xenobiotic and endobiotic compounds. This antioxidant enzyme system detoxifies chemical compounds containing an electrophilic group, using the reduced glutathione (GSH) as cofactor:

$$GSH + CDNB \rightarrow GS\text{-}DNBconjugate + HCl$$

GST were determined at 340 nm using 1-chloro-2,4 dinitrobenzene (CDNB) as substrate ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). The assay was carried out in 100 mM potassium phosphate buffer (pH 6.5), 1.5 mM CDNB, and 1.5 mM GSH.

Glutathione reductase (GR), also known as glutathione-disulphide reductase (GSR), catalyses the reduction of glutathione disulphide (GSSG) to the sulfhydryl form glutathione (GSH) which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell. GR use the NADPH as cofactor to make the reaction occurs:

$$GSSG + NADPH \rightarrow 2GSH + NADP^+$$

GR activity was measured by following the oxidation of NADPH at 340 nm during the reduction of GSSG (extinction coefficient, $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). The assay conditions were 100 mM potassium phosphate buffer (pH 7.0), 1 mM GSSG and 60 μM NADPH.

Glutathione peroxidase (GPx) is an antioxidant system that is composed of glutathione dependent enzymes. GPx catalyse the reduction of organic and inorganic peroxides to the corresponding alcohol, using GSH as cofactor. Peroxides reduction takes place via glutathione transformation from its reduced form to the oxidized one:

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O \quad \text{organic peroxides}$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \quad \text{inorganic peroxides}$$

The formed GSSG is reconverted to GSH by GR with NADPH consumption:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The activity of both Se-dependent and Se-independent GPx forms were measured in a coupled enzymatic assay, where GSSG is converted to the reduced form GSH. The consumption of NADPH was measured as decrease of absorbance at $\lambda = 340$ nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM

dithiothreitol (DTT), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.8 mM cumene hydroperoxide as substrate.

In order to obtain the specific antioxidant activities, data were normalized with the relative protein concentration according to Lowry method (1951) with bovine serum albumin used as standard.

6.2.3.2 Total oxyradical scavenging capacity (TOSC) assay

Polychaetes were homogenized on ice (1:10 w:v) using a glass plotter in 100 mM K-phosphate buffer (pH 7.5) containing NaCl (1.5%), PMSF (0.1 mg mL^{-1}) and protease inhibitors as aprotinin (0.008 TIU mL^{-1}), leupeptin (1 μg mL^{-1}), pepstatin (0.5 μg mL^{-1}). After centrifuging at 100,000 x g for 70 min at 4 °C, cytosolic fractions were collected and maintained at -80 °C until they were used for the analysis.

The analysis of the total oxyradical scavenging capacity (TOSC) is a reliable tool for quantitatively assessing the biological resistance to toxicity of different forms of ROS, including peroxyl radicals, hydroxyl radicals and peroxynitrite decomposition products (Winston et al. 1998, Regoli and Winston 1999). The assay is based on the capability of cellular antioxidants to reduce the oxidation of α-keto-γ-methiolbutyric acid (KMBA) in presence of artificially generated oxyradicals. KMBA is oxidized to ethylene gas, and the time-course of its formation is monitored during the whole assay duration by gas-chromatographic analyses (measured at 10-12 min time intervals). The antioxidant efficiency of a sample is quantified by its ability to scavenge the produced oxyradicals thus inhibiting their reaction with KMBA and ethylene formation (Winston et al. 1998) (Fig. 6.1). TOSC assay was performed resorting to different forms of artificially oxidants generated at constant rate: peroxyl radicals (ROO•), hydroxyl radicals (HO•) and peroxynitrite (HOONO) (Fig. 6.1). Peroxyl radicals were obtained by the thermal decomposition of 2,2'-azo-bis-(2-methylpropionamide)-dihydrochloride (ABAP) according to Winston and Cederbaum (1983); hydroxyl radicals were generated thanks to the iron-ascorbate Fenton reaction; peroxynitrite were generated from 3-morpholinosydnonimine (SIN-1) following the procedure described by Lomonosova et al. (1998). Final assay conditions were: (i) 0.2 mM KMBA, 20 mM ABAP in 100 mM potassium phosphate buffer (pH 7.4) for peroxyl radicals; (ii) 1.8

mM Fe^{3+}, 3.6 mM EDTA, 0.2 mM KMBA, 180 mM ascorbic acid in 100 mM potassium phosphate buffer (pH 7.4) for hydroxyl radicals; and (iii) 0.2 mM KMBA and 80 mM SIN-1 in 100 mM potassium phosphate buffer (pH 7.4) with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) for peroxynitrite (Regoli 2000). Reactions were conducted at 35 °C in 10 ml vials sealed with gas-tight Mininert valves (Supelco, Bellefonte, PA) in a final volume of 1 ml (Fig. 6.1). Aliquots of 200 ml of ethylene gas formed were taken from the head space of reaction vessels with a gas-tight syringe at 10–12 min intervals during the time course of the reaction. Then, they were injected into the gas chromatography instrument equipped with a Supelco SPB-1 capillary column (30 m × 0.32 mm × 0.25 μm) and a flame ionization detector (FID) (Regoli 2000). The oven, injection and FID temperatures were maintained constant at respectively, + 35, + 160 and + 220 °C; hydrogen flux of 30 mL min^{-1} and Helium flux of 3 mL min^{-1}. Each sample, according to this procedure, was analysed every 15 min, for 105 min.

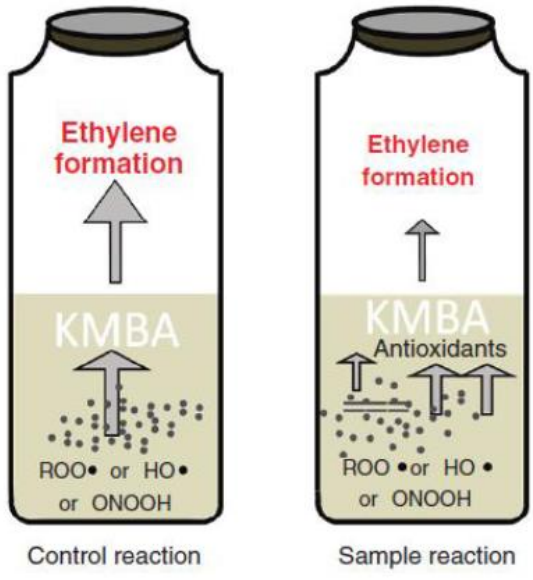

Figure 6.1 Ethylene formation inside the vials of control reaction and sample reaction (Gorbi and Regoli 2012).

The TOSC value was calculated using the formula:

$$TOSC = 100 - (\int SA / \int CA \times 100)$$

Where ∫SA and ∫CA are the integrated areas calculated under the kinetic curve produced during the reaction course for sample (SA) and control (CA) reactions, respectively (Winston et al. 1998). The experimental TOSC value falls between 0 and 100. In case of the absence of oxyradical scavenging capacity, ethylene formation is not reduced as compared to control ($\int SA / \int CA = 1$) resulting in a TOSC value equal to zero. Alternatively, the total inhibition of ethylene formation throughout the assay (SA = 0) would corresponds to a theoretical maximum TOSC value of 100. In order to obtain the specific TOSC value, data were normalized with the relative protein concentration determined according to Lowry et al. (1951) method using bovine serum albumin as a standard.

6.2.4 Statistic analyses

Results of the background analyses on the antioxidant enzymes activity were tested using PERMANOVA non parametric test with square root transformation and 1,000 number of permutations. For the background results of the total oxyradical scavenging capacity (TOSC) assay was applied a two way analysis of variance (2 way ANOVA) test with: 'origin/species' (confronted factors) as fixed factor with two levels (Castello Aragonese as putative *P. massiliensis* and Sant'Anna as putative *P. dumerilii*) to test the effect of different native pH conditions or putative different species; 'time' fixed factor with three levels (April, October, February), nested in 'origin/species', to test responses temporal variation between specimens. Tukey's honest significance test was used as multiple comparison test procedure at the 95% of confident interval. The assumption of normal distribution was tested with the Shapiro-Wilk test, whereas the Bartlett's test was used to verify that data met the assumption of homogeneity.

The effects of the exposure to different pH treatments in the laboratory experiment on the antioxidant enzymes activity and on the total oxyradical scavenging capacity were tested using a two ways analysis of variance (2 way ANOVA) with: 'origin/species' (confronted factors) as fixed factor with two levels (Castello Aragonese as putative *P. massiliensis* and Sant'Anna as putative *P. dumerilii*) to test the effect of

different native pH conditions or putative different species; fixed factor ‘pH treatment’ with three levels (normal, low pH, extreme low pH), nested in ‘origin/species’, indicated the pH treatment at which specimens were exposed to. Tukey’s honest significance test was used as multiple comparison test procedure (with 95% family-wise confidence level) to test for significance differences between the enzymes activity values and TOSC values at different pH treatments, after the translocation experiment. The Shapiro-Wilk test was used to confirm that both enzymes and TOSC data met the assumption of normal distribution and those who did not, were square root transformed. The assumption of homogeneity, tested with Bartlett’s test, was also met. All statistical analyses were performed using RStudio (RStudio Team 2015) and Primer v 6 (Clarke and Gorley 2006).

6.3 Results

Results of the background antioxidant enzyme activity statistic analyses, based on origin/species x time, suggest that no significant differences occur with the exception of GR; differences in enzyme activities are recorded only considering time factor (Tab. 6.2 and 6.3). Catalase exhibits the highest values in April in both species, followed by a significant decrease in February in putative *Platynereis dumerilii*; CAT activity in putative *P. massiliensis* is stable throughout the period-examined (Tab. 6.2, Fig. 6.2). A significant difference according to the species factor is also recorded for the CAT activity (factor origin/species, F = 4.2082, df = 1, p = 0.042) (Tab. 6.3). A similar trend, with the highest activity in April, is also observed for glutathione S-transferases in both species, while the lowest values are recorded in October (*Platynereis dumerilii*) and February (*P. massiliensis*); even in this case the enzymatic activity is more constant in the vent population (Tab. 6.2, Fig. 6.2). The glutathione reductase activity is significantly reduced in putative *Platynereis dumerilii* from April to February (origin/species x season, F = 20.346, df = 2, p = 0.001), while the highest values in the vent population are exhibited in October (Tab 6.2, Fig. 6.2). The activity of the Se-dependent and Se-independent GPx in both species is higher in October and decreases in February and April (Tab. 6.2, Fig. 6.2).

TOSC values, toward different ROS, change significantly only in peroxynitrite (HOONO) (origin/species x time, F = 6.8292, df = 2, p = 0.006), for which the influence of the time fixed factor is also observed (Tab. 6.3). The efficiency in neutralize peroxyl radicals is highest in April for Sant'Anna population (putative *Platynereis dumerilii*) with a slight decrease in the following months (October and February); in *P. massiliensis* the highest value is recorded in October but the TOSC activity is kept constant over the other periods considered (Tab. 6.2, Fig. 6.2). For the hydroxyl radicals, the highest efficiency is detected for putative *Platynereis massiliensis* in October and the lowest one in February; on the other hand, for putative *P. dumerilii* the highest activity is recorded in April, but values do not show strong variations over the periods considered (Tab. 6.2, Fig. 6.2). About the peroxynitrite, the highest neutralizing efficiency is detected in Octorber in both species; the lowest value is observed in February for the normal pH population, while no marked temporal variations are

highlighted in the vent population (putative *Platynereis massiliensis*) (Tab. 6.2, Fig. 6.2).

Table 6.2 Basal antioxidant enzymes activities and total oxyradical scavenging capacity values in putative *Platynereis dumerilii* and *P. massiliensis*. Values are expressed as μmol min^{-1} mg^{-1} protein for CAT; nmol min^{-1} mg^{-1} protein for GST, GR and GPx; TOSC units/mg protein for ROO•, HO• and ONOOH; in paretheses the values of standard deviation.

	April 2016		**October 2016**		**February 2017**	
	P. dum	*P. mass*	*P. dum*	*P. mass*	*P. dum*	*P. mass*
CAT	52.91 (15.88)	48.63 (20.33)	35.52 (1.85)	44.82 (1.95)	24.05 (6.91)	45.66 (9.78)
GST	58.58 (24.73)	37.75 (12.10)	29.62 (4.63)	34.24 (5.05)	30.44 (8.58)	26.59 (5.62)
GR	54.53 (25.45)	21.68 (2.06)	30.43 (6.94)	32.11 (5.94)	6.98 (3.47)	22.67 (5.15)
GPx	13.91 (6.20)	16.30 (3.01)	26.29 (13.67)	23.29 (4.64)	20.32 (5.39)	18.05 (3.97)
ROO•	1044.88 (359.44)	918.83 (331.44)	904.99 (203.17)	988.68 (312.71)	774.35 (146.88)	946.79 (270.06)
HO•	654.36 (240.36)	640.78 (181.19)	581.23 (157.36)	748.18 (186.85)	647.05 (180.70)	446.85 (89.39)
HOONO	500.79 (127.41)	451.32 (142.57)	786.50 (101.88)	522.71 (134.84)	336.42 (37.99)	507.98 (139.86)

Table 6.3 PERMANOVA (enzymes) and 2-way ANOVA (TOSC) tests for significant differences between different months of the year (April, October, February) in the background analyses. Significant codes: $p \leq 0.001$ '***', $p \leq 0.1$ '**', $p \leq 0.05$ '*', $p > 0.05$ 'ns'.

Biomarker	**species x time**	**x species**		**x time**
CAT	ns	**	> *P. massilienisis*	***
GST	ns	ns		***
GR	***	ns		***
GPx	ns	ns		***
ROO•	ns	ns		ns
HO•	ns	ns		ns
HOONO	***	ns		***

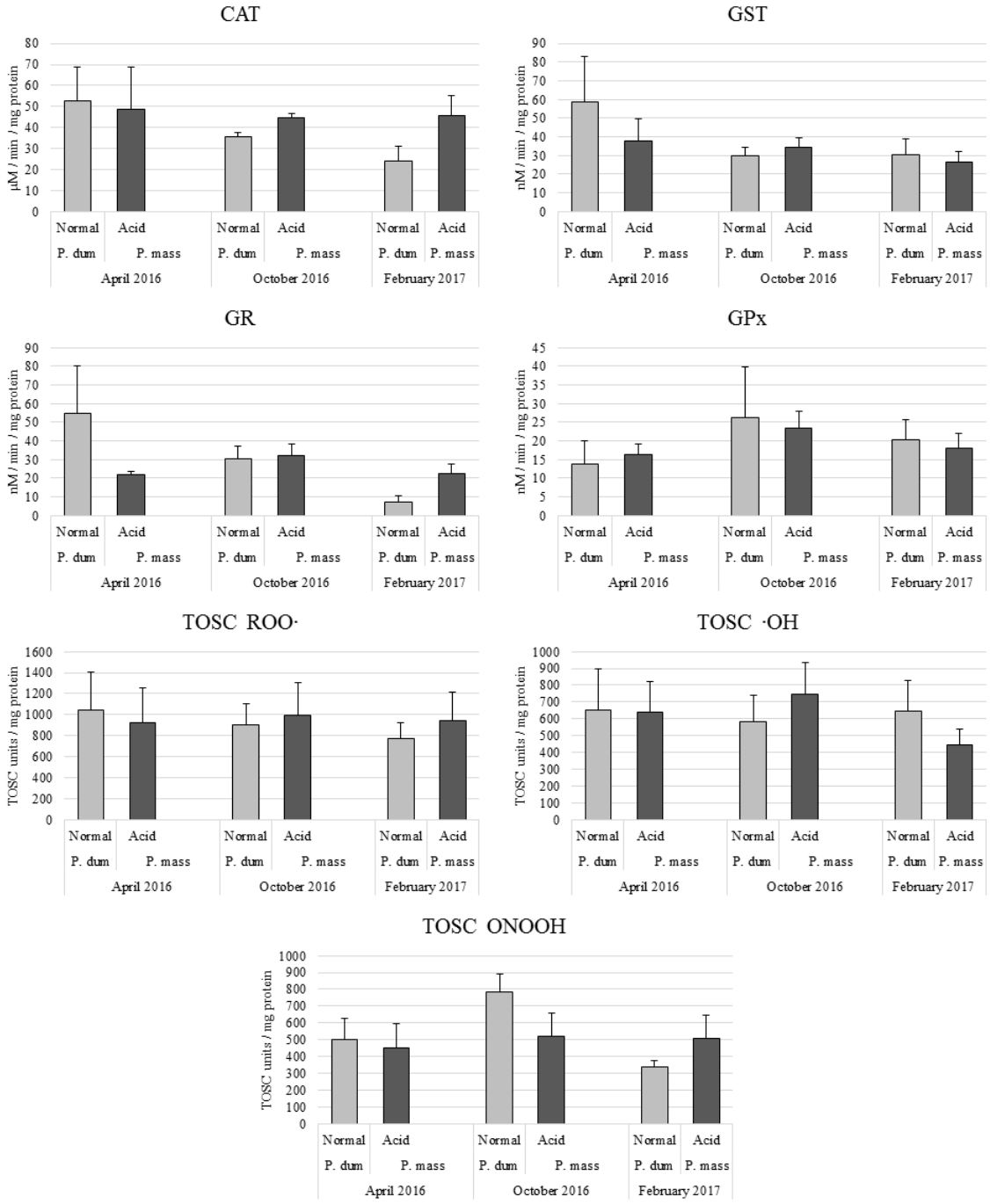

Figure 6.2 Basal antioxidant enzymes activities (CAT, GST, GR, GPx) and total oxyradical scavenging capacity (TOSC) toward peroxyl radicals (ROO•), hydroxyl radicals (HO•) and peroxynitrite (ONOOH) in putative *Platynereis dumerilii* and *P. massiliensis*. Values are expressed as µmol min^{-1} mg^{-1} protein for CAT; nmol min^{-1} mg^{-1} protein for GST, GR and GPx; TOSC units/mg protein for ROO•, HO• and ONOOH.

Regarding the laboratory pH experiment, the number of survivors and percentages of mortality after the translocation experiment of 30 days of exposure to different pH conditions, are reported in Table 6.4.

Table 6.4 Number of live individuals at retrieval / total number of individuals at the beginning of the experiment (in parentheses the percentage of mortality).

Putative species	Normal pH	Low pH	Extreme low pH
Platynereis dumerilii	44/75 (42.5%)	49/75 (35.1%)	26/75 (65.3%)
Platynereis massiliensis	42/90 (53.3%)	53/90 (41.1%)	49/90 (46.1%)

Among antioxidant defences, no significant differences are highlighted by the 2-way ANOVA test (origin/species x pH treatment); significant dissimilarities ($p \leq 0.05$) are recorded in the activity of each antioxidant enzyme according to the origin/species fixed factor, and for CAT and GPx activities according to the pH treatment fixed factor (Tab. 6.6). The CAT highest values in both species are detected in the extreme low pH treatment (Tab. 6.5, Fig. 6.3). Glutathione S-transferases activity shows different trends between the two species: in putative *Platynereis dumerilii* the highest value is reached with normal pH while the lowest one with low pH treatment; conversely, in putative *P. massiliensis* the enzyme activity becomes higher passing from low pH, normal to extreme low pH (Tab. 6.5, Fig. 6.3). The GR activity is higher in control conditions in *Platynereis dumerilii* (normal pH) and then decreases with the acidic treatments; in *P. massiliensis* the enzyme efficiency increases passing from low pH, normal to extreme low pH (Tab. 6.5, Fig. 6.3). The activity of the Se-dependent and Se-independent GPx is higher with acid extreme pH treatment, while the lowest efficiency is detected in low pH conditions in both species (Tab. 6.5, Fig. 6.3). The putative *Platynereis dumerilii*, living under control/normal pH conditions (Sant'Anna rocks) shows an overall higher efficiency in the antioxidant enzyme activities when compared to the putative *P. massiliensis* thriving inside the vent area, with the sole exception for GR (Tab. 6.5, Fig. 6.3).

TOSC values toward different ROS do not significantly differ based on origin/species x pH treatment (Tab. 6.6). Conversely, considering only the species factor, *Platynereis dumerilii* and *P. massiliensis* show the highest TOSC values against peroxyl radicals in normal pH, and against hydroxyl radicals in extreme low pH

conditions (Tab. 6.5, Fig. 6.3). The efficiency in neutralizing peroxynitrite gradually increases in *Platynereis dumerilii* moving towards the more acidic pH conditions; conversely, it reaches the maximum value in normal pH conditions in *P. massiliensis* (Fig. 6.3).

Table 6.5 Antioxidant enzymes activities and total oxyradical scavenging capacity values in putative *Platynereis dumerilii* and *P. massiliensis* after the translocation experiment of 30 days with three different pH conditions (normal, low and extreme low pH).Values are expressed as µmol min^{-1} mg^{-1} protein for CAT; nmol min^{-1} mg^{-1} protein for GST, GR and GPx; TOSC units/mg protein for ROO•, HO• and ONOOH; in paretheses the values of standard deviation.

	Platynereis dumerilii			*Platynereis massiliensis*		
	Normal - cc	Low	Extreme low	Normal	Low - cc	Extreme low
CAT	52.24 (3.50)	50.14 (13.98)	56.02 (6.13)	36.12 (8.38)	37.29 (8.97)	52.18 (5.55)
GST	50.26 (9.34)	37.78 (6.02)	46.39 (7.96)	29.40 (10.07)	26.05 (5.31)	36.31 (4.09)
GR	21.28 (4.35)	15.46 (1.77)	16.00 (5.06)	27.37 (1.86)	24.91 (0.65)	29.59 (5.61)
GPx	23.26 (9.85)	14.24 (7.20)	47.57 (3.76)	15.22 (8.68)	10.39 (3.14)	29.55 (2.45)
ROO•	1364.16 (256.19)	1044.32 (316.64)	1247.66 (656.93)	1478.52 (142.19)	1166.13 (92.84)	900.51 (201.23)
HO•	741.84 (130.76)	666.39 (82.01)	903.89 (315.90)	751.54 (94.02)	753.92 (164.94)	937.68 (191.74)
HOONO	609.80 (91.48)	700.39 (95.65)	823.94 (410.03)	687.86 (6.19)	551.92 (140.07)	587.00 (71.71)

Table 6.6 2-way ANOVA test for significant differences between pH treatments after the 30 days laboratory experiment. Significant codes: $p \leq 0.0001$ '****', $p \leq 0.001$ '***', $p \leq 0.1$ '**', $p \leq 0.05$ '*', $p > 0.05$ 'ns'.

Biomarker	**species x pH treatment**	**x species**		**x pH treatment**
CAT	ns	**	> *P. dumerilii*	*
GST	ns	***	> *P. dumerilii*	ns
GR	ns	****	> *P. massiliensis*	ns
GPx	ns	**	> *P. dumerilii*	***
ROO•	ns	ns		ns
HO•	ns	ns		ns
HOONO	ns	ns		ns

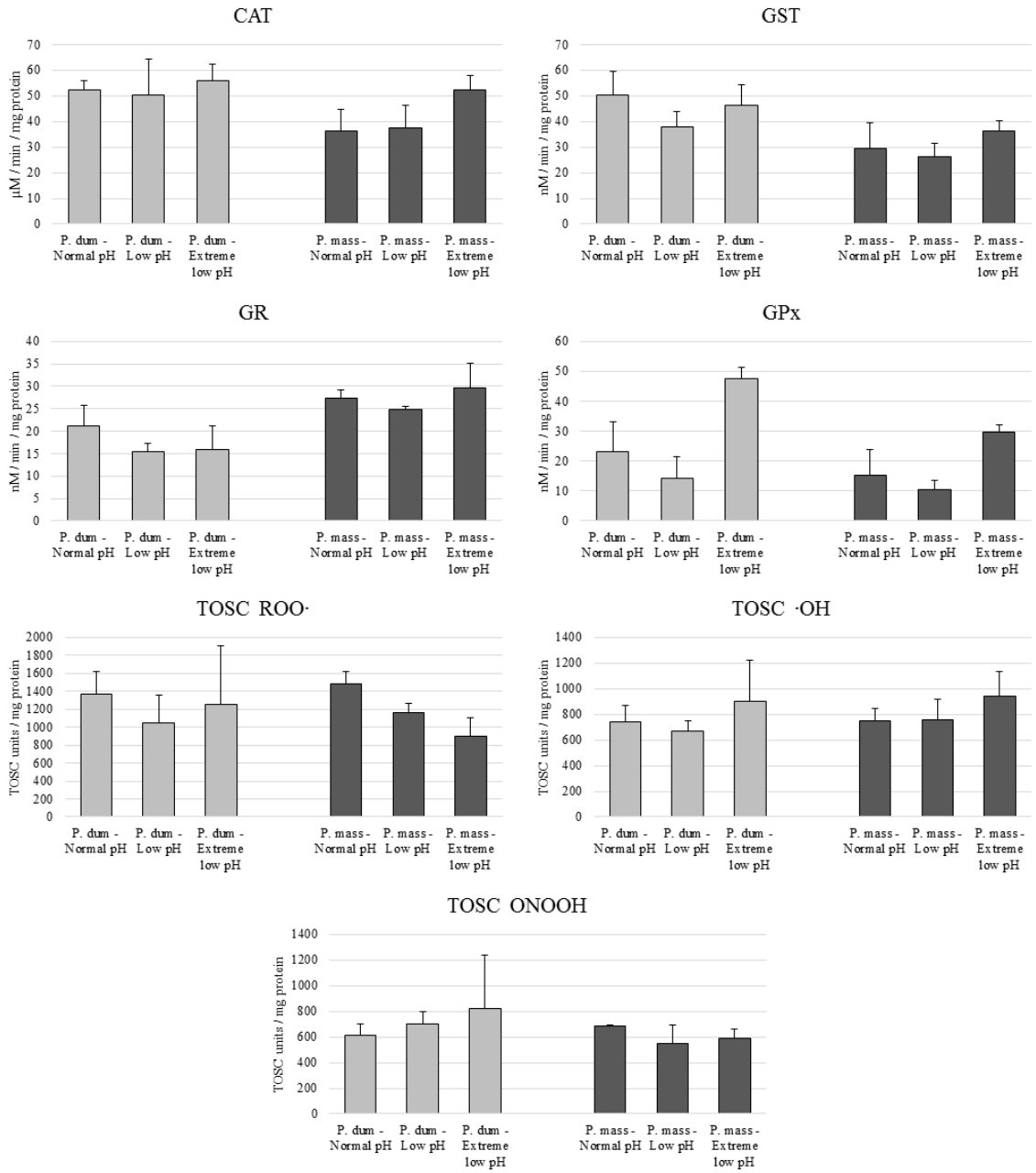

Figure 6.3 Antioxidant enzymes activities (CAT, GST, GR, GPx) and total oxyradical scavenging capacity (TOSC) toward peroxyl radicals (ROO•), hydroxyl radicals (HO•) and peroxynitrite (ONOOH) in putative *Platynereis dumerilii* and *P. massiliensis* after the translocation experiment of 30 days with three different pH conditions (normal pH, low pH, extreme low pH).Values are expressed as µmol min^{-1} mg^{-1} protein for CAT; nmol min^{-1} mg^{-1} protein for GST, GR and GPx; TOSC units/mg protein for ROO•, HO• and ONOOH.

6.4 Discussion

The elevated pCO_2 acts as a prooxidant stressor with several effects already described in marine organisms, such as increased production of ROS and consequent alteration on many processes at the cellular level (Tomanek et al. 2011, Zhang et al. 2011, Matoo et al. 2013, Matozzo et al. 2013, Vehmaa et al. 2013). Based on the specific pH tolerance of the two *Platynereis* siblings highlighted in previous studies (Calosi et al. 2013b, Ricevuto et al. 2015a), it would be interesting to evaluate whether differences exist in the basal antioxidant defence system efficiency, explaining the ability of *P. massiliensis* to prevail in acidified conditions. It would also be informative to evaluate whether the oxidative effect, caused by decreased pH levels and chronic OA conditions, affects both species equally.

We have characterized the basal levels of antioxidant defence systems of the two populations/siblings on a temporal scale, considering that different times and seasonal related conditions (mainly temperature) influence the physiology of organisms with profound effects on their metabolic activities. On a temporal basis, no differences are highlighted between species in both antioxidant enzyme activities and TOSC, with the exception of glutathione reductase and peroxynitrite (see Table 6.2). Comparing the two species/populations, regardless the time of collection, the only significant difference recorded is for the CAT activity, with a higher overall efficiency in *Platynereis massiliensis*. In general, the highest mean values of both enzymatic activities and TOSC are detected during April for *Platynereis dumerilii* and October for *P. massiliensis*; the lowest ones in February for *P. dumerilii* and in April for *P. massiliensis*. These figures suggest that the two populations/species can have different temporal seasonal-related trends in the antioxidant defence systems probably related to life and reproductive cycles. A comparison with the temporal trend antioxidant defence system basal levels of other temperate marine organisms has been made with: the European eel *Anguilla anguilla*, the stripped mullet *Mugil cephalus* from the Obertello lagoon (Tuscany, Italy) (Gorbi et al. 2005), and the mussel *Mytilus galloprovincialis* from the area of Portonovo (Ancona, Northern Adriatic sea) (Bocchetti snd Regoli 2006). These studies reveal a response variation associated with seasonality of both environmental and biological factors, such as temperature and reproductive cycles.

Our *Platynereis* species are characterized by higher values of antioxidant activity when compared to the above mentioned species, with the exception of peroxynitrite in both *Anguilla anguilla* and *Mugil cephalus*. The *Platynereis dumerilii* temporal trend is more variable when compared to its sibling *P. massiliensis*, indicating that the species needs to modulate its redox responses in time to keep the oxidative stress level of its tissues under control. The ability of *Platynereis massiliensis* to maintain stable levels of antioxidant defences, regardless of the period of the year, might represent a winning strategy that allows it to live and dominate in the highly selective conditions of low pH/elevated pCO_2. To better understand the trend of the antioxidant defence systems variability would be informative in order to extend the background analyses of both populations to the four seasons of the year (with at least two sampling dates per season).

The translocation experiment under laboratory controlled conditions is a preliminary study in which the two species, putative *Platynereis dumerilii* and *P. massiliensis*, were exposed to three different pH treatments for 30 days. The rather high mortality rates recorded might be explained as a biological response of the organisms to different pH values, especially for *P. dumerilii* under constant extreme low pH conditions (7.40 ± 0.05). The tough pH regime, that was set for the whole duration of the experiment, does not correspond to the condition that can be found in an open system, such as CO_2 vents in which the pH is subjected to high fluctuations even on a daily interval (see Kroeker et al. 2011). The mortality percentages observed for both species under laboratory conditions are even higher than those recorded during an *in situ* reciprocal transplant experiment (see Ricevuto et al. 2015a), and this could probably be due to the prolonged exposure to constant low or extreme low pH values. Secondly, the specimen collection and manipulation might have represented an additional source of stress, although this was also the case for transplants under *in situ* conditions. The acidification does not significantly affect the species antioxidant defences, even if *Platynereis massiliensis* exhibits its greatest antioxidant defence systems efficiency mainly in extreme low pH conditions (with the exception of ROO• and ONOOH). Regardless the pH treatment, the CAT, GST and GPx antioxidant efficiency is higher in *Platynereis dumerilii*, while *P. massiliensis* shows a more active GR. Considering 'origin/species' nested in 'pH treatment', the results of the experiment do not reveal a more marked antioxidant efficiency for one species compared to the other one, conversely to what has been previously observed by Ricevuto et al. (2015a)

for TOSC activity in an *in situ* experiment. The authors stated that the native vent population (putative *Platynereis massiliensis*) highlighted more elevated basal antioxidant efficiency towards ROO• and ONOOH, suggesting the need for greater antioxidant protection in conditions of chronic exposure to oxidative stress compared to the control population (putative *P. dumerilii*) (Ricevuto et al. 2015a). Based on our findings, it can be concluded that the greater intraspecific efficiency of putative *Platynereis massiliensis* (vent population) antioxidant defence systems in extreme low pH conditions (CAT, GST, GR, GPx, TOSC •OH) might be interpreted as a signal of its higher success in the colonization and survival of acidified habitats (Lucey et al. 2015).

Our statistically not significant results suggest two possible hypotheses: (i) the acidic pH conditions, considered in both background analyses and laboratory experiment, are not high enough to warrant this kind of antioxidant responses (enzymes and TOSC); (ii) due to the close proximity between the study sites (only 600 m apart), it is possible that the control area of Sant'Anna has been colonized over time by individuals of the *Platynereis* spp. population which dominates the acidified areas of Castello Aragonese, resulting in an antioxidant answers homogenization between the two populations.

Chapter 7

Discussion and Conclusions

7.1 General discussion

The oceans and the atmosphere are parts of the same dynamic system in which they are strictly correlated and influenced by each other. Since the beginning of the Industrial Revolution, the increasing concentration of greenhouse gases (GHG) in the atmosphere, in particular carbon dioxide (CO_2), has been responsible for the notorious phenomenon of Global Climate Change (GCC). As a result of the continuous exchanges between oceans and atmosphere, an equivalent rise of dissolved CO_2 in seawater has lead, and is still leading, to a pH drop off in a process termed Ocean Acidification (OA). An increasing number of studies focusing on those organisms considered the most susceptible to the seawater pH decrease, such as calcifiers, has been recorded over the last few decades. However, the responses of a large compartment of the benthic communities still need to be investigated (Fabricius et al. 2014). For non-calcifying organisms, in which the effect of OA might be more difficult to detect, a multidisciplinary approach that considers different aspects of the biology of model organisms represents the best strategy. In the present PhD thesis, a multidisciplinary approach was followed in order to study the Evo-Devo model polychaete species *Platynereis dumerilii* and its sibling *P. massiliensis*, with the aim of evaluating the potential effects of OA as a driver of genetic differentiation and phenotype/genotype selection. In order to reach the above mentioned goal, eight populations of *Platynereis* spp. were sampled in different geographical areas located along the Italian coasts and characterized by different pH conditions (acidified *vs* normal). The multidisciplinary approach was based on the analysis of each population from different points of view: (i) morphological and morphometric; (ii) reproductive biology and gamete morphology; (iii) population genetics and phylogeography; (iv) population genomics; (v) ecotoxicology.

The morphological and morphometric approaches highlight differences in the mean body biomass among populations with the exception of: Levante Bay *vs* Ponente Bay, Levante Bay *vs* Castello Aragonese, Sant'Anna *vs* Ponente Bay, Santa Caterina *vs* La Spezia. Contrasting results with previous studies (Calosi et al. 2013b, Garilli et al. 2015) have been detected in Ischia, where specimens that thrive under low pH conditions (Castello Aragonese) show a larger body size when compared to specimens from the control site (Sant'Anna). These differences are exclusively attributable to a pH

effect, being the temperature of the two study sites exactly the same. The larger mean biomass of La Spezia specimens can be due to lower temperature conditions, which are translated as decreased metabolic rates and increased body size (Gillooly et al. 2001, Bickford et al. 2010). Regardless of the pH conditions, a sort of dwarfism effect has been highlighted for insular populations (Ischia and Vulcano) likely due to resource limitation mechanisms in spatially constrained environments (as observed for mammals by Lomolino 1985). No dissimilarities are detected in the morphological comparison (parapodia shape, homogomph falciger notochaetae shape and paragnaths arrangement) among populations from acidified *vs* normal pH areas, and the morphology of the observed characters is consistent with some of the previous species descriptions (Day 1967, Hartmann-Schröder 1996, Vietez et al. 2004). The morphometric comparison between individuals of *Platynereis dumerilii* (dominant in Blue Bay) and *P. massiliensis* (dominant at Castello Aragonese) belonging to the same size range, reveals few significant statistical dissimilarities, mainly in the dorsal cirrus length of the IV and XIII chaetigers from the prostomium and at the 10^{th} body segments from the pygidium, suggesting the occurrence of few morphometric differences between the two sibling species.

The life history and reproductive biology approach highlights two different reproductive strategies among the studied *Platynereis* populations, consistent with the previously reported descriptions of *P. dumerilii* and *P. massiliensis* reproductive habits (Hauenschild 1951, Schneider et al. 1922, Fischer et al. 2010, Helm et al. 2014). The predominance of the brooding reproductive strategy, typical of *Platynereis massiliensis*, in the acidified areas confirmed the ability of this species to thrive in low pH conditions (Lucey et al. 2015). Several hypotheses may explain this peculiar distribution. A first explanation is a pH-driven selection of brooding tolerant phenotypes able to adapt to chronic OA conditions. The CO_2-dominating brooding *Platynereis massiliensis* can also be competitively favoured by the unique conditions of the vent area ('chemical island' effect), which may represent a sort of barrier for pelagic larval stages of the free spawner *P. dumerilii* (Lucey et al. 2015). The brooding reproductive strategy might be the winning one in stressful conditions. The habit of this species, which lives attached to macroalgal thalli, coupled with the brooding inside the tube, could favour its resistance to OA. The photosynthesis of macroalge and the microhabitat conditions inside the tube

may buffer the negative effect of high pCO_2/low pH, thus facilitating the embryos' survival as observed for other invertebrates (Saderne and Wahl 2012, Wahl et al. 2017). The coexistence of specimens with both reproductive strategies in some of the studied areas (Ponente Bay - Vulcano, Panarea and Santa Caterina) confirms the sympatric nature of the sibling species. The cross-breeding experiments suggest a reproductive isolation between vent populations due to the brooding habit/semi-direct development (less dispersal potential) and the elevated geographical distances among sampling sites. The mature spermatozoa morphology (TEM and SEM observations) is consistent with previous studies (Pfannenstiel et al. 1987, Lücht and Pfannenstiel 1989); the spermatozoa structure of the broadcasting *Platynereis dumerilii* (rounded nucleus) is considered as primitive or ect-aquasperm type, while the *P. massiliensis* sperm (elongated nucleus) is an aberrant or ent-aquasperm type (Jamieson and Rouse 1989, Lücht and Pfannenstiel 1989).

The population genetics approach, through to the amplification of the mitochondrial DNA COI marker, allows the identification of four different species/clades: clades 1 and 2 that mainly group individuals collected from acidified sampling sites, clades 3 and 4 that mainly include specimens sampled in control/normal pH areas. The significant average genetic distances calculated between clades (1-2: 19.9%; 3-4: 16.8%) as well the inclusion in the analyses of COI reference sequences (Boore and Brown 2000, Lucey et al. 2015), suggest the occurrence of two complexes of sibling species: clades 1-2 attributed to *Platynereis massiliensis* and its sibling; clades 3-4 attributed to *P. dumerilii* and its sibling; supporting what was previously observed by Wäge et al. (2017) on a reduced set of populations analysed. For both species, a comparison with the COI reference sequences from the type locality would be desirable (*Platynereis massiliensis* from Marseille and *P. dumerilii* from La Rochelle, France). The samples grouping inside each clade do not seem to be directly correlated with the different geographical origin, with the sole exception of the Levante Bay population whose individuals clustered all together in clade 2. Santa Caterina specimens form a subclade in both clades 1 and 4 likely due to the occurrence of a geographical barrier (this is the only site in the Eastern Mediterranean), which reduces the gene flow between Tyrrhenian and Ionian populations, as already observed for other marine organisms with high motility (*Mullus barbatus* Maggio et al. 2009, *Alexandrium minutum* Casabianca et al. 2011). It is unsurprising that the brooding species *Platynereis*

massiliensis, with low dispersal potential, shows isolated populations and increased genetic diversity (Palumbi et al. 1994) as highlighted for other direct developer invertebrates species; e.g. the nereidid polychaetes *Hediste japonica* (Sato and Tsuchiya 1991, Sato and Masuda 1997) and *Neantes acuminata* (Reish et al. 2014), as well as two marine isopods species (Teske et al. 2007). As discussed in Chapter 3, the overall predominance of the *Platynereis massiliensis* complex in acidified conditions might be correlated with more susceptible pelagic larval stages to low pH (Dupont et al. 2008, Kurihara 2008, Byrne 2011) and a buffering effect made by the parental tube of brooding species and/or by the photosynthesis carried out by algae in which brooding polychaetes live (Saderne and Wahl 2012, Wahl et al. 2017). Population genetic results are consistent with reproductive biology observations with the exception of: (i) Castello Aragonese population which includes few individuals belonging to the free spawner clade, while the laboratory rearing highlighted only brooding behaviour; (ii) Sant'Anna population which is divided between the brooder clades and the free spawner one (clade 4), conversely only free spawners were observed from the laboratory rearing.

The population genomics approach, thanks to a RAD-seq protocol application, confirms the occurrence of two different complexes of sibling species which do not hybridize to each other and that are putatively identified as *Platynereis dumerilii* and *P. massiliensis*. Each complex consists of at least two different subclades whose composition is not strictly correlated with the geographical origin, and without signs of gene flow. In both species, the individuals from the Santa Caterina (Lecce) population are genetically differentiated from the other members of each species-complex likely due to the accumulation of mutations caused by the high geographical distance between the Tyrrhenian and Ionian Seas (Frankham et al. 2002). Although both species' occurrence do not appear statistically correlated with the original pH habitat conditions, a low pH preference for specimens identified as *Platynereis massiliensis* (66% of the individuals were sampled in acidified areas) and a normal pH preference for those identified as *P. dumerilii* (85.7% of the individuals were collected in normal pH areas) has been recorded. The reasons for *Platynereis massiliensis* success in acidified areas are those as discussed above. Surprisingly, inside *Platynereis massiliensis* complex, the two nearby Ischia populations of Castello Aragonese and Sant'Anna (approximately 600 metres apart) show high levels of genetic differentiation (85.58%).

This high differentiation might be caused by: the co-occurrence of two different siblings of *Platynereis massiliensis* in Ischia; a 'founder effect' caused by limited numbers of colonists which settled in the Castello Aragonese vent area; the brooding nature of *P. massiliensis* which implies a reduced dispersal potential (Beckwitt 1980, Schneider et al. 1992). The high differentiation of *Platynereis massiliensis* populations can also be translated as a greater ability of this species to adapt to different habitats.

The ecotoxicological approach aims to compare the antioxidant capacity of the two putative sibling species, *Platynereis dumerilii* (Sant'Anna population, normal pH) and *P. massiliensis* (Castello Aragonese population, low pH), and to evaluate the oxidative effect of low pH conditions on both of them. Both aims have been reached through background analyses of basal levels of antioxidant defence systems based on temporal scale (three periods considered), and by a 30-day laboratory experiment under controlled conditions with different pH treatments (normal, low pH and extreme low pH). The background analyses highlighted no significant differences between putative *Platynereis dumerilii* and *P. massiliensis* in both antioxidant enzymes activity and TOSC, with the exception of glutathione reductase (GR) and peroxynitrite (ONOOH). *Platynereis dumerilii* shows high temporal variability and it seems to be able to modulate its redox responses in time keeping the oxidative stress level of its tissues under control. *Platynereis massiliensis* maintains stable levels of antioxidant defences independently from the temporal scale; this might represent a winning strategy that allows it to succeed in low pH/elevated pCO_2 conditions. When compared with other temperate marine organisms, the antioxidant activities are generally highest in *Platynereis* spp. specimens (Gorbi et al. 2005, Bocchetti et al. 2006). Preliminary results of the translocation experiment show no significant differences between the two species/populations based on the different pH treatments, conversely to what has been previously observed by Ricevuto et al. (2015a) in an *in situ* experiment. The putative *Platynereis massiliensis* (vent population) shows a greater intraspecific efficiency of antioxidant defence systems in extreme low pH conditions (CAT, GST, GR, GPx, TOSC •OH) which might be the reason for its higher success in the colonization of acidified habitats.

7.2 Conclusions

This PhD thesis project focuses on the study of two polychaete sibling species, *Platynereis dumerilii* and *P. massiliensis*, from the phenotypic and genomic points of view in order to evaluate the potential effect of Ocean Acidification (OA) in driving their genetic differentiation and possible genotype/phenotype selection. The multidisciplinary approach based on different kinds of analyses (morphological and morphometric; life history traits and reproductive biology; population genetics/phylogeography; population genomics; ecotoxicology) has allowed the following general conclusions to be reached.

The species putatively identified as *Platynereis dumerilii* and *P. massiliensis* represent two different complexes of sibling species that do not hybridize to each other and that are composed of at least two different species' entities each (clades). The species seem to be morphologically indistinguishable at the sexually immature adult stage, even if few morphometric differences have been detected comparing two of the studied populations (*Platynereis dumerilii* from Blue Bay and *P. massiliensis* from Castello Aragonese). The life history traits, reproductive biology and gamete morphology of the two sibling complexes are completely different. Individuals identified as *Platynereis dumerilii*, mainly collected from normal pH areas (Blue Bay), show the typical broadcasting behaviour, semelparous reproduction, epitokous transformation and primitive/ect-aquasperm type. On the other hand, specimens putatively belonging to *Platynereis massiliensis*, mainly sampled in the CO_2 natural vent systems (Ischia and Vulcano), display a brooding behaviour, protandrous hermaphroditism and iteroparous reproduction with aberrant/ent-acquasperm type. In four study areas of this thesis (Sant'Anna - Ischia, Ponente Bay - Vulcano, Panarea and Santa Caterina) both species with contrasting reproductive strategies were observed validating the sympatric origin of the siblings. The low pH conditions of the acidified vent systems do not show a direct effect on the genotype selection and speciation of the *Platynereis dumerilii* and *P. massiliensis* sibling complexes. Nevertheless, low pH/elevated pCO_2 seems to be a driving factor which indirectly contributes to the *Platynereis massiliensis* success under OA conditions: (i) brooding tolerant phenotype able to adapt to chronic OA conditions; (ii) limitation of the *P. dumerilii* pelagic larval stages thrive (chemical barrier); (iii) a sort of buffering effect made by the brooding

parental tube which might create a microhabitat with stress-free conditions; (iv) a buffering effect made by the macroalgae photosynthetic activity in which polychaetes live. Further experiments to investigate whether effectively low pH conditions favour brooding reproductive strategies, rather than the broadcasting ones, would be necessary. The *Platynereis massiliensis* complex shows high levels of genetic populations' differentiation likely due to the low dispersal capacity of this semi-direct developer species, even between nearby areas such as Castello Aragonese and Sant'Anna (Ischia). This elevated genetic differentiation might suggest a greater potential of this species to adapt to different habitats. In fact, conversely to *Platynereis dumerilii* in which the antioxidant efficiency varies over temporal scale, the antioxidant defence systems of *P. massiliensis* remain more stable over time and the highest intraspecific efficiency is detected in extreme low pH conditions. The higher stability and greater responsiveness to hypercapnia conditions might explain the ability of *Platynereis massiliensis* to persist in such stressful conditions.

Considering that the target species of this PhD project, in particular *Platynereis dumerilii*, are biological models used in many laboratories, a proper identification of the complexes of sibling species gives important information for further studies in different fields, such as Evo-Devo, ecology, physiology and genomics. Furthermore, the characterization of local populations is essential to detect potential ongoing cryptic speciation processes and to evaluate areas of endemism, with fundamental implications for management and conservation. Due to the morphological similarities between the two sibling complexes, *Platynereis massiliensis* seems to have been largely overlooked and previous records of *P. dumerilii* should be reconsidered in light of this 'sibling problem'. It is possible that the *Platynereis dumerilii* considered by Bellan (1980) as a bioindicator of polluted conditions off the Marseille coast (French Mediterranean coast; type locality *P. massiliensis*), could have been *P. massiliensis*. Considering that the two species are morphologically indistinct (see Chapter 2), they could be easily confused also by highly expert polychaete taxonomists. Complexes of sibling species hold great promise for testing current evolutionary theories in ecology since they facilitate comparative studies due to their few differences. *Platynereis* siblings might be used to compare different biological processes and patterns to the true *P. dumerilii*/*P. massiliensis* genetic strains, contributing to increase knowledge of the effects of marine climate change stressors on the biology of marine organisms.

7.3 Future perspectives

This PhD thesis provides a broad overview of the phenotypic and genomic variability of the polychaete sibling species *Platynereis dumerilii* and *P. massiliensis* and the possible relationships with ocean acidification. However, in order to improve this work, would be interesting:

- To extend the morphometric analysis to a larger number of individuals genetically identified as *P. dumerilii* and *P. massiliensis*, taking into account additional measured body features;
- To compare the oocytes morphology and ultrastructure (trough TEM and SEM observations) of species/populations;
- To include in the population genetics analysis COI species sequences from type localities to effectively confirm their belonging to *P. dumerilii* and *P. massiliensis*;
- To repeat the same RAD-seq approach with more species samples per population in order to better evaluate a putative pH effect of the species/populations differentiation;
- To perform antioxidant background analyses on a more intense temporal scale comparing previously genetically identified populations as *P. dumerilii* and *P. massiliensis*.

Furthermore, in order to strengthen these findings, further studies are suggested:

- Transcriptomics refers to the study of the complete set of transcripts or transcriptome (mRNA, ncRNA, rRNA, tRNA, and other ncRNAs) for a given developmental stage or physiological condition (Wang et al. 2009). The transcriptome varies with developmental stage, physiological condition, and external environment and its analysis represents a powerful tool to quantify change in the expression levels for each gene among different transcriptome samples, to map the transcriptome and to determine the functional structure of each gene in the genome (Costa et al. 2010, Ruan et al. 2004, Wang et al. 2009). Based on these premises, the use of a transcriptomic approach to compare early life and adult stages of putatively *P. dumerilii* and *P. massiliensis* specimens

collected from normal and low pH habitats respectively (Blue Bay - La Spezia and Castello Aragonese - Ischia), could give researchers important information about the transcriptomic response to CO_2-driven seawater acidification (variation in gene expression and functions).

- As the field of OA has grown, researchers have increasingly turned to laboratory experiments to understand the impact of increased pCO_2 on marine organisms. However, other changes such as ocean warming and deoxygenation are currently occurring, coupled with the increasing of pCO_2. Benthic marine invertebrates live in a multi-stressor habitat and they often experience different environmental conditions during their life phases (Byrne and Przeslawski 2013). In order to better understand the impacts of anthropogenic changes, a multi-stressors laboratory experiment with independent regulation systems for CO_2 concentration, temperature and O_2 levels should give interesting outputs. Multi-stressor experiments must incorporate ecological control, extreme natural and extreme predicted conditions, to provide useful information about the impact of global climate change.

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Appendix 1

CTAB method for gDNA extraction

DNA isolation

1) Grind the whole polychaete in a 1.5 ml tube
2) Add 150 µl of pre-warmed CTAB buffer and 10 µl of proteinase K (20 mg mL^{-1}) and vortex
3) Incubate at 60°C for 1 hour mixing by inversion every 10-15 minutes
4) Add 300 µl of 24:1 chloroform:isoamyl alcohol and mix to emulsify

   This produces two phases, an upper aqueous phase that contains the DNA, and a lower chloroform phase that contains some degraded proteins, lipids, and many secondary compounds. The interface between these two phases contains most of the 'junk' – cell debris, many degraded proteins, etc.
5) Centrifuge 20 minutes at 15°C at 14000 rpm
6) Transfer the aqueous (upper) layer ~150 µl to a new clean tube

Precipitation

7) Add 600 µl of cold isopropanol stored at -20°C and mix gently to precipitate nucleic acids
8) Place the tubes at -80°C for 1 hour or at -20°C overnight
9) Centrifuge 30 minutes at 4°C at 14000 rpm
10) Pour off supernatant carefully

Ethanol Washing

11) Resuspend nucleic acid pellet in 600 µl of absolute EtOH at RT
12) Centrifuge 5 minutes at 4°C at 14000 rpm
13) Remove the absolute EtOH and air dry sample for ~30 minutes
14) Resuspend in 30 µl of Milli-

Appendix 2

RAD-seq protocol

Restriction

1) Digest 44 µL of genomic DNA (500 ng) for each of the 50 samples with 1.0 µL *Sbf*I-HF restriction enzyme and 5.0 µL of the respective 10X SmartCut Buffer; 50 µL total reaction volume
2) Incubate at 37 °C for 60 min
3) Heat-inactivate the restriction enzyme at 80 °C for 20 min and wait 30 min at RT

P1 Adapter Ligation

Ligate barcoded, restriction site overhang-specific P1 adapters onto complementary compatible ends on the genomic DNA created in the previous step.

4) To create Adapters P1, dilute each of the 50 oligos from 40 µM to 100 nM.
5) To allow the Adapters P1 ligation add the following: 3.0 µL of Adapter P1 (100 nM), 1.0 µL 10X SmartCut Buffer, 0.6 µL rATP (100 mM), 0.5 µL concentrated T4 DNA Ligase (2,000 U/mL), 4.9 µL H_2O; 60.0 µL total volume
6) Incubate for 40 min at RT
7) Heat-inactivate T4 DNA Ligase at 65 °C for 20 min. Allow reaction to cool slowly to ambient temperature before shearing (30 min at RT)

Sample Multiplexing and DNA Shearing

8) Combine barcoded samples in an equal ratio (approx. 20 ng per each sample)
9) Clean up using AMPure XP Beads
10) Check the fragments size with a Bioanalyzer system with High Sensitivity DNA kit (Agilent Technologies)
11) Shear DNA samples to an average size of 400 bp to create a pool of P1-ligated molecules with random, variable ends, with the Covaris S2 system using Covaris mircoTUBEs (cat# 520045) in a volume of 130 µl for shearing with the following setting: duty cycles 5%, intensity 3, cycles/burst 200, duration 125 s
12) Check if the fragments size is in the expected range with a Bioanalyzer system with High Sensitivity DNA kit (Agilent Technologies)

13) Reduced the reaction volume to 56 µL with the SpeedVac concentrator (approx. 30 min at 30 °C)

End repair

NEBNext Ultra DNA Library Prep Kit for Illumina.

14) Add the following components to the 56 µL of fragmented DNA and mix: 3.0 µL End Prep Enzyme Mix, 6.5 µL End Repair Reaction Buffer (10X); 65.5 µL total volume
15) Incubate at 20 °C for 30 min, at 65 °C for 30 min

P2 Adapter ligation

NEBNext Ultra DNA Library Prep Kit for Illumina.

16) Add the following and mix: 15 µL Blunt TA Ligase Mater Mix, 3.0 µL Adapter P2 (10 µM), 1.0 µL Ligation Enhancer; 84.5 µL total volume
17) Incubate at 20 °C for 20 min

Size selection

NEBNext Ultra DNA Library Prep Kit for Illumina.

18) Approximate insert size: 400-500 bp. 1st Bead Selection: add 35 µL of beads; 2nd Bead selection: add 15 µL of beads
19) Elute the DNA target with 20 µL H_2O

RAD-tag Amplification/Enrichment

In this step high-fidelity PCR amplification is performed on P1 and P2 adapters ligated DNA fragments, enriching for RAD tags that contain both adaptors.

20) Determine DNA concentration with the Qubit dsDNA HS Assay kit designed for the Qubit Fluorimeter (Life Technologies)
21) In a thin-walled PCR tube combine: 25 µL Buffer Q5, 1.0 µL primer Forward (25 µM), 1.0 µL primer Reverse (25 µM), 4.0 µL RAD library template (50 ng), 19 µL H_2O; 50 µL total volume. Perform 16 cycles of amplification in a thermal cycler: 30s 98 °C, 16 x (10 s 98 °C, 75 s 65 °C), 5 min 65 °C, hold 10 °C

22) Clean up PCR amplification with AMPure XP Beads and elute DNA target in 20 µL H_2O
23) Determine again DNA concentration with the Qubit dsDNA HS Assay kit designed for the Qubit Fluorimeter (Life Technologies) to verify if the amplification occur

Sequence library on an Illumina HiSeq 2000 platform

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REAPPRAISAL OF *PLATYNEREIS MASSILIENSIS* (MOQUIN-TANDON) (ANNELIDA, NEREIDIDAE), A NEGLECTED SIBLING SPECIES OF *PLATYNEREIS DUMERILII* (AUDOUIN & MILNE EDWARDS)

PLATYNEREIS MASSILIENSIS *(MOQUIN-TANDON) (ANNELIDA, NEREIDIDAE), UNA SPECIE CRIPTICA DIMENTICATA DI* PLATYNEREIS DUMERILII *(AUDOUIN & MILNE EDWARDS)*

Abstract **-** *Specimens of the putative polychaete species* Platynereis dumerilii *(Audouin & Milne Edwards) (Nereididae), collected from the acidified areas of the Castello Aragonese CO_2 vents system of the Ischia island, when reproduced in the laboratory, gave evidence on the occurrence of the sibling species,* Platynereis massiliensis *(Moquin-Tandon). This taxon has been neglected in our seas, since it can be distinguished from its sibling only by the reproductive/life history habit (brooding/semi-direct development, protandrous hermaphrodite). With this work we reappraised the presence of* P. massiliensis *along the Italian coast, reporting a stable population at Ischia and preliminary data on its reproductive biology.*

Key-words: *Polychaeta, sibling species, reproductive biology, ocean acidification, Tyrrhenian Sea.*

Introduction - The polychaete *Platynereis massiliensis* (Moquin-Tandon, 1869) (Annelida, Nereididae) is known as the sibling species of *Platynereis dumerilii* (Audouin & Milne Edwards, 1834), whose adult, not reproductive, stages are identical but with a highly different reproductive habit/life history (Hauenschild, 1951). *P. dumerilii* in fact, is gonochoric, with heteronereid modification (epitoky), a single reproductive event in life (semelparous), free spawning and pelagic/planktotrophic larval stage; while the sibling *P. massiliensis* shows no epitokous transformation and is a protandrous hermaphrodite, characterized by egg brooding and semi-direct larval development (Schneider *et al.*, 1992) (Tab. 1). Due to its peculiar reproduction and phylogenetic vicinity to *P. dumerilii*, also *P. massiliensis* is used as a model species for basic biology and Evo-Devo studies (Fischer and Dorresteijn, 2004; Helm *et al.*, 2014). In the Mediterranean Sea *P. massiliensis* was first described in the Marseille region (type locality), and the original description is quite poor and incomplete, and lacking the figure. The species was then reported in the Gulf of Naples by Hauenschild (1951) - a work where the species is also better described and compared with its sibling -, and in Banyuls-sur-Mer (France) by Schneider *et al.* (1992). However, due probably to the fact that these studies were dealing with embryology and larval development, and considering also that the Hauenschild's work (1951) was in German, they were not taken into account in the taxonomic/ecological literature and in benthic investigations. In fact, *P. massiliensis* is not reported in Mediterranean polychaete check-lists and revisions (Arvanitidis, 2000; Viéitez *et al.*, 2004; Çinar *et al.*, 2014; Mikac, 2015), including those considering the Italian coasts (Castelli *et al.*, 2008; Mikac, 2015). Ecological and monitoring surveys, generally based on the analysis of fixed adult, and not-reproductive, specimens, recorded only the presence of *P. dumerilii*, which is therefore considered the most common and widespread species of the genus *Platynereis* in the whole Mediterranean Sea.

Studies of polychaete populations in naturally acidified waters of the Castello Aragonese CO_2 vent system at the Ischia Island, revealed that *P. dumerilii* represents one of the dominant species in the most acidified zones of the vents (Kroeker *et al*., 2011; Ricevuto *et al*., 2012, 2014). Therefore, this species was selected to perform eco-physiological investigations to study acclimatization and adaptation to the unique acidified conditions of this system. Studying a putative *P. dumerilii* population sampled in the acidified areas of the CO_2 vents at Ischia, Calosi *et al.* (2013) identified a distinct genotype, which clustered separate from the actual *P. dumerilii* genetic clade. Subsequently, Lucey *et al.* (2015), rearing specimens from the same population in laboratory conditions, observed egg brooding inside of the tube, and proved that this different genotype, selected against the acidic waters, belonged to the sibling *P. massiliensis*. The aim of this work is to reappraise the occurrence of this species along the Italian coast, and provide preliminary observations on the reproductive biology of the Ischia CO_2 vent's population.

Tab. 1 - Main reproductive features of the two *Platynereis* sibling species: *P. dumerilii* and *P. massiliensis*.

Principali caratteristiche della biologia riproduttiva delle due specie sibling di Platynereis*:* P. dumerilii *and* P. massiliensis.

Platynereis dumerilii	***Platynereis massiliensis***
Gonochoric	Protandrous hermaphrodite
Semelparous	Iteroparous
Epitoke (heteronereis stage)	Atoke (no heteronereis stage)
Free spawner	Brooder inside the tube
Oocyte size <180 μm	Oocyte size >250 μm
Planctotrophic larvae	Lecitotrophic eggs, semi-direct larval development

Materials and methods - Specimens of *Platynereis* spp. were collected, from May to October 2014, associated to the macroalgae *Halopteris scoparia* and *Dictyota* spp. settled on the rocky reefs and on the dead *Posidonia oceanica* seagrass matte of the acidified south side areas of the CO_2 vents system off the Castello Aragonese (Ischia), where the species represents one of the dominant polychaetes (Kroeker *et al*., 2011; Calosi *et al*., 2013; Ricevuto *et al*., 2014). To study the reproductive biology, specimens were reared in Petri bowls (100 ml, approx. 5 specimens per bowl) using filtered sea water (0.22 μm), and kept under early summer controlled conditions (21±1 °C and L:D = 16h:8h) inside a thermostatic chamber; fresh spinach was used to feed the worms. Fresh filtered sea water and food supply were provided approx. every week, when specimens were also checked for their reproductive status.

Results and conclusions - A few specimens of *Platynereis* sampled in mid-May 2014 (n=3), mid-June (n=2) and end of October (n=2), were observed to laid eggs inside their tubes after being kept in laboratory conditions for a period ranging between one to four weeks (Fig. 1). The eggs had a size of 350 μm diameter, and were oxygenated by ventilation movements of the female inside the tube. The eggs hatched from one to two weeks after being laid. The juveniles remained inside the parental tube up to 5-6 segments; at 9-10 segments they started to build their own tubes (Fig. 1). We followed the growth of approx. 80 juveniles in total; after 4 months (August-November) only 12 survived, reaching 10-18 mm length.

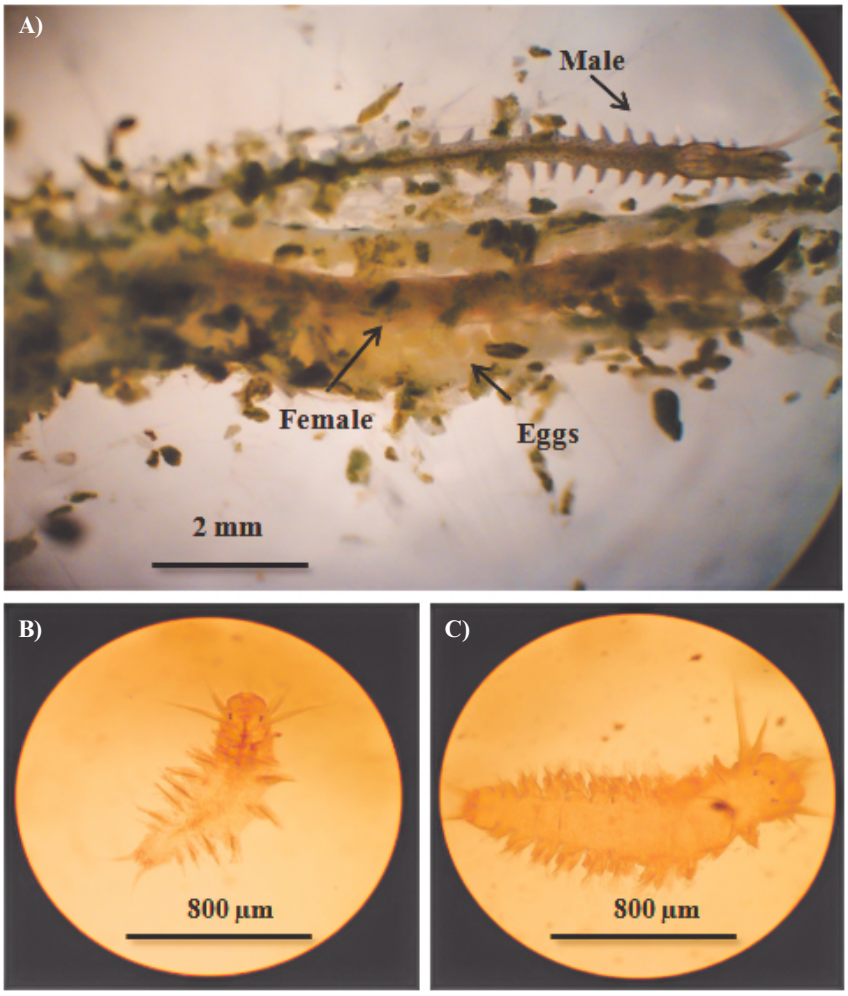

Fig. 1 - A) Male and female specimens of *P. massiliensis* with eggs deposited inside the tube (arrows); B) juvenile of *P. massiliensis* with 7 chaetigers; C) juvenile with 10 chaetigers.

A) Individui maschile e femminile di P. massiliensis *con uova deposte all'interno del tubo (frecce); B) individuo giovanile di* P. massiliensis *con 7 chetigeri; C) individuo giovanile con 10 chetigeri.*

We further demonstrated that *P. massiliensis* dwells preferentially in the acidified zones of the Castello vent's system where the pH can reach values below 7.0 units (Calosi *et al*., 2013; Ricevuto *et al*., 2014). The unique distribution of this species opens challenging questions about the potential advantage of the brooding habit as adaptation to stressed habitats, such as those submitted to ocean acidification (Lucey *et al*., 2015). The occurrence of *P. massiliensis* in our seas is reappraised, since Hauenschild's record in 1951 in the Gulf of Naples (Mergellina), not far from Ischia, and therefore this species should be included in the checklist of the Italian marine fauna (Castelli *et al*., 2008). In the light of these results, the actual distribution of *P. dumerilii* needs also to be reconsidered since its neglected sibling species could be more common and widespread than expected (Wäge *et al*., submitted; Valvassori and Gambi, unpublished data).

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ORIGINAL PAPER

The sibling polychaetes *Platynereis dumerilii* and *Platynereis massiliensis* in the Mediterranean Sea: are phylogeographic patterns related to exposure to ocean acidification?

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Abstract High pCO_2 environments, such as volcanic carbon dioxide (CO_2) vents, which mimic predicted near-future scenarios of ocean acidification (OA), offer an opportunity to examine effects of low pH conditions on marine biodiversity and adaptation/acclimatization of marine organisms to such conditions. Based on previous field studies in these systems, it is predicted that the stress owing to increasing CO_2 concentrations favours the colonization by invertebrate species with a brooding habit. The goal of this study was to investigate the relative occurrence of the two sibling species *Platynereis dumerilii* (Audouin & Milne-Edwards, 1834) (free spawner) and *Platynereis massiliensis* (Moquin-Tandon, 1869) (egg brooder) in two shallow CO_2 vents off Ischia and Vulcano islands (Italy, Tyrrhenian Sea), and in various areas with ambient pH conditions, where they represent one of the dominant genera. Phylogeographic analyses were integrated with reproductive biology and life-history observations on some selected populations thriving in the vent areas. This approach revealed the presence of four distinct *Platynereis* clades. Whereas two clades primarily inhabit CO_2 vents and are presumably all brooders, the other two clades dominate the non-acidified sites and appear to be epitokous free spawners. We postulate that one of the brooding, vent-inhabiting clades represents *P. massiliensis* and one of the free spawning, non-vent-inhabiting clades represents *P. dumerilii*, although confirmation of the species status with sequence data from the respective-type localities would be desirable.

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Introduction

The increase of atmospheric carbon dioxide (CO_2) levels since the industrial revolution, has contributed to the phenomenon known as 'ocean acidification' (OA) (IPCC 2013). Increased pCO_2 in seawater leads to the formation of carbonic acid, which then releases hydrogen ions, lowering the pH of the water (Gattuso and Hansson 2011). From the industrial revolution to the present time, oceanic pH has dropped from 8.2 to 8.07, and a further pH drop by 0.3–0.4 pH units is predicted by the year 2100 (Gattuso and Lavigne 2009; Caldeira and Wickett 2003). The focus of research on the potential impacts of OA has initially resided on potentially vulnerable stress-intolerant species (Fischlin et al. 2007), mainly calcifiers depending on calcium carbonate for the growth of their shells and tests. Much less information is available about non-calcifying organisms, which climate change could affect also at the physiological

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(acclimatization) or genetic level (adaptation) (Calosi et al. 2013; Harvey et al. 2014).

Volcanic CO_2 vents represent useful model systems and natural laboratories to investigate short as well as long-term effects of OA on benthic biota and sea-floor ecosystems (Hall-Spencer et al. 2008; Fabricius et al. 2011). Polychaetes are a dominant group in the benthic communities in these systems, especially in temperate areas such as the island of Ischia (Gambi et al. 2016) (Castello Aragonese vent's area; Kroeker et al. 2011) and Vulcano island (Levante Bay vent's area; Vizzini et al. 2017) in Italy (Figs. 1, 2). Therefore, polychaetes are appropriate models to address various aspects of acclimatization/adaptation to OA, such as settlement pattern (Ricevuto et al. 2014), and assemblage responses along pH gradients (Gambi et al. 2016), functional traits analyses (Lucey et al. 2015, 2016; Gambi et al. 2016), trophic habit and acclimatization (Calosi et al. 2013; Ricevuto et al. 2015a), and biochemical responses to OA stress (Turner et al. 2015; Ricevuto et al. 2015b, 2016).

Platynereis dumerilii (Audouin and Milne-Edwards, 1834) is a non-calcifying annelid worm of the family Nereididae (see Vieitez et al. 2004 for a morphological description). This meso-herbivore species (Gambi et al. 2000; Ricevuto et al. 2015a) has a semelparous reproduction, with a breeding period that occurs in the Mediterranean Sea between May and September (Giangrande et al. 2002). The breeding period begins with a "sexual metamorphosis" during which an immature, benthic atokous individual transforms in a sexually mature pelagic epitokous form called heteronereis (Fischer and Dorresteijn 2004). During metamorphosis, animals increase their eyes size, subdivide their trunk into two parts with different shapes of parapodia,

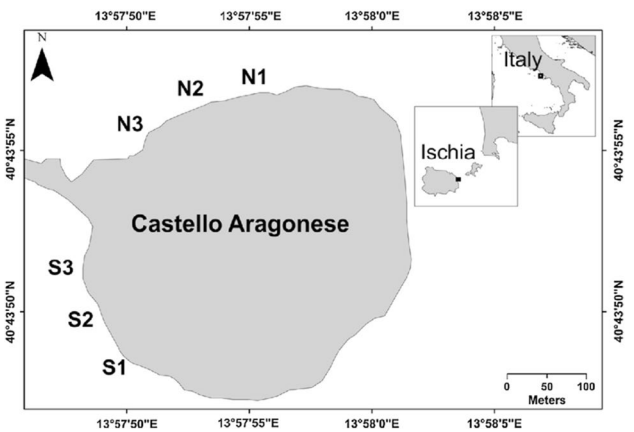

Fig. 1 Map of Castello Aragonese study area (Ischia island, Italy) with location of the sampling stations on the north and south sides along a pH gradient (N1, N2, N3–S1, S2, and S3) (from Ricevuto et al. 2014). pH values measured with in situ sensors by Kroeker et al. (2011) are as follows: N1 = 8.0 ± 0.1; N2 = 7.8 ± 0.2; N3 = 7.2 ± 0.4; S1 = 8.1 ± 0.1; S2 = 7.8 ± 0.3; and S3 = 6.6 ± 0.5

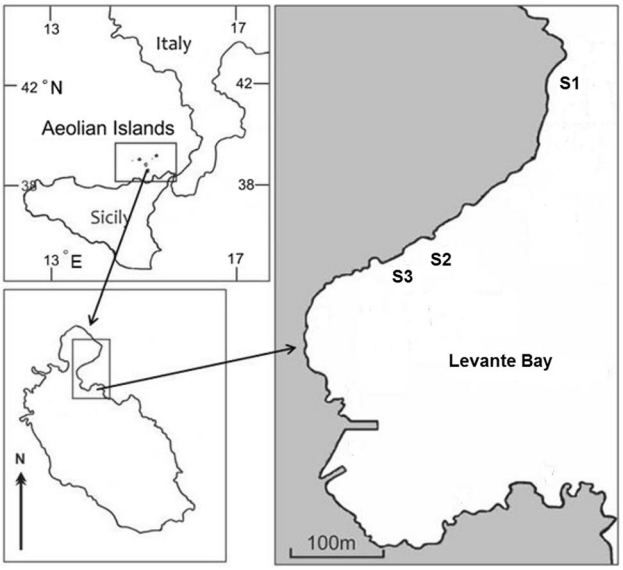

Fig. 2 Map of the Levante Bay study area (Vulcano island, Italy), with location of the sampling stations along a pH gradient (S1, S2, and S3) (from Johnson et al. 2013). pH mean values measured with in situ sensors by Johnson et al. (2013) area as follows: S1 = 8.18; S2 = 8.05; and S3 = 7.49

and develop sexually dimorphic body coloration. Mature pelagic heteronereids swim rapidly and attract individuals of the opposite sex through the release of sex pheromones (Zeeck et al. 1988; Fischer and Dorresteijn 2004). Swarming culminates in the nuptial dance, with sexual partners rapidly swimming in a circle and delivering their gametes into the water column, leading to the fertilization of the eggs. After spawning, males and females die (Fischer and Dorresteijn 2004).

Platynereis dumerilii is a well-understood Evo-Devo model species especially for comparative studies, since its evolutionary lineage has been slow-evolving (Zantke et al. 2014). Highly conserved gene structure and cell types with protein sequences, as well as the position and the number of introns in its genome, show low rates of divergence from vertebrates as opposed to other faster evolving species (Raible et al. 2005; Zantke et al. 2014). *P. dumerilii* is also considered a bioindicator of organic pollution (Bellan 1980). It has been reported as one of the most abundant species from the vegetated rocky reefs of natural CO_2 vents off Ischia Island (Italy) in the Mediterranean Sea (Kroeker et al. 2011; Ricevuto et al. 2014). Calosi et al. (2013) found that the population inhabiting the naturally acidified water of the CO_2 vents at Ischia was genetically and physiologically distinct from nearby non-acidified control population. Genetic distances between the vent-inhabiting lineages and those from nearby non-acidified control locations suggested that they represent different sibling species (Calosi et al. 2013). The habitat preference of the two sibling species is

not absolute; however, occasional individuals of the presumably vent-adapted lineage appeared in control environments and vice versa. Calosi et al. (2013) surmised that these individuals may not actually reproduce in the mismatched environments and that competitive exclusion insured that they only formed a small portion (1:10–1:15) of the respective population. Lucey et al. (2015), reared specimens in the lab and observed egg brooding in the vent-inhabiting population, indicating that it represents likely the only sibling species of *P. dumerilii* known to date, *P. massiliensis* (Moquin-Tandon, 1869). These two sibling species are morphologically indistinguishable as immature adults but are easily identified upon reproduction. In contrast to *P. dumerilii*, *P. massiliensis* shows no epitokous transformation and mature worms are protandric hermaphrodites, characterized by egg brooding inside the tube, lecithotrophic larval stages with a semi-direct development, and egg hatching into juveniles within the parental tube (Hauenschild 1951; Helm et al. 2014; Lucey et al. 2015).

Sibling species represent cryptic sister species that are the closest relative of each other and have not been morphologically, and therefore, taxonomically distinguished (Bickford et al. 2007).

Here, we analyze phylogeographic patterns among populations from two CO_2 vent systems (Ischia and Vulcano in the Souther Tyrrhenian Sea) and various non-acidified control sites in the Mediterranean Sea and the Atlantic Ocean in conjunction with the reproductive modes of the vent-inhabiting lineages. This integrated approach aims to shed light on the presence and relative proportion of the sibling species in relation with OA and to check for the occurrence of possible selected genotypes and other cryptic species.

Materials and methods

Field collection of *Platynereis* spp. specimens

Morphologically identified *P. dumerilii* specimens were collected in two different CO_2 vent systems located in Ischia island (Bay of Naples, Italy) and Vulcano island (Aeolian Archipelago, Tyrrhenian Sea, Italy) (Figs. 1, 2). The vent system of Ischia island includes both the South and North sides of the Castello Aragonese, a small islet of volcanic origin connected by an artificial bridge to the main Island of Ischia on the north-eastern side. The continued CO_2 (90–95%) gas emissions from the shallow waters have created pH gradients on both sides of the Castello Aragonese based on which six areas with different pH, respectively, three on the north and three on the south, can be distinguished (N1, N2, N3–S1, S2, and S3) (Fig. 1). Sites numbered 1 are designated as control stations with ambient pH values (mean values N1 = 8.0 ± 0.1, S1 = 8.1 ± 0.1), sites numbered 2 are considered intermediate pH stations (N2 = 7.8 ± 0.2, S2 = 7.8 ± 0.3), while sites numbered 3 are considered as the most acidified ones (N3 = 7.2 ± 0.4, S3 = 6.6 ± 0.5; Kroeker et al. 2011; Ricevuto et al. 2014).

Specimens were collected in the two most acidified areas of the south side, S3 and S2, and in the most acidic one of the north side, N3 (e.g., Calosi et al. 2013; Ricevuto et al. 2014) (Fig. 1). For additional pH and carbonate chemistry data of these vents, see Ricevuto et al. (2014).

In the vent area, the dominant taxon is *P. massiliensis* (Calosi et al. 2013; Lucey et al. 2015); however, most specimens were not mature, so it was impossible to examine gametes. In addition, various swimming heteronereids (*P. dumerilii* epitokous specimens in reproduction) were collected over the south acidified areas S3 and S2 of the Castello at night (11.00 PM–1.00 AM, 24 May 2011; Larsson T., Gambi M.C. & Hardege J. personal observation) with long-handed nets from a rubber-boat with a strong light to attract the worms (Hardege et al. 1990). Since the depth at the south acidified areas is no more than 3 m, we assume that the pH on the surface is similar to the pH near the bottom. In this zone, the pH shows relatively high temporal and spatial variability, with mean values ranging according to the season from 7.75 to 7.69 in S2 and from 7.32 to 6.59 in S3 (see Ricevuto et al. 2014 for data pH overview). The collected specimens were immediately transferred into RNALater solution (Sigma-Aldrich Company Ltd., Gillingham, UK) on the boat and stored at −20 °C for genetic analyses.

The shallow Levante Bay, situated on the eastern side of Vulcano island, was used as an additional naturally acidified study area, since the main vent system (primary vents) of the island (lat 38°25′01″N and long 14°57′36″E) occurs there at less than 2 m depths (Boatta et al. 2013) (Fig. 2). Similar to Ischia, this vent system has also been partitioned into different sites (S1, S2, and S3) along a pH gradient, at different distances from the primary vents (Fig. 2) (Johnson et al. 2013). *Platynereis* specimens were collected in the most acidified station S3 (mean pH 7.49) in early May 2013. Further specimens were collected in other geographic areas away from the influence of the vents to check for the possible presence of the sibling species. Sampling sites out of the vents were located around Ischia, at various distances from the vent areas and in the Gulf of Naples (Italy): Sant' Anna rocks (Ischia), San Pietro (Ischia), Forio (Ischia), Nisida (Gulf of Naples); in some areas of the Western Mediterranean: Palinuro (Tyrrhenian Sea), Ustica island (Tyrrhenian Sea), STARESO Belgian Marine Station at Calvi (Corsica, France), Blanes (Catalunia, Spain); in the Eastern Mediterranean: Santa Caterina (Ionian Sea, Eastern Mediterranean, Italy), and in the North Atlantic: Arcachon (Atlantic, France), and Bristol (Atlantic, UK; see Table 1).

Worms were collected by either snorkeling or SCUBA diving (at 1–3 m depth) by detaching thalli of macroalgae (in

Table 1 Details of collection sites of *Platynereis* spp. populations considered in this study, and number of individuals successfully sequenced and used for the COI tree

Collection location	Latitude/longitude	Acidified/ Control	Collection date	Distance from Castello vents (Ischia)	*n*. individual sequenced
Castello S2/S3, Ischia (south side)	40° 43′ 51.18″N; 13° 57′47.45″E	A	07/07/2010	0	12
Castello S2/S3 (heteronereis stage)	40° 43′ 51.18″N; 13° 57′47.45″E	A	5/24/2011	0	10
Castello N3, Ischia (north side)	40° 43′ 55.00″N; 13° 57′48.82″E	A	6/19/2014	0	4
S. Anna, Ischia	40° 43′ 35.76″N; 13° 57′ 36.95″E	C	11/21/2011	600 m	14
S. Pietro, Ischia	40° 44′ 47.59″N; 13° 56′ 39.86″E	C	11/17/2011	4 km	16
Forio, Ischia	40° 44′ 25.08″N; 13° 51′ 41.54″E	C	5/20/2012	8 km	7
Nisida island, Gulf of Naples, Italy	40° 46′ 32.60″N; 14° 09′ 45.52″E	C	11/14/2011	18 km	15
Palinuro, Tyrrhenian Sea, Italy	40° 01′ 52-76″N; 15° 16′ 08.74″E	C	6/29/2013	250 km	8
Levante Bay S3, Vulcano, Italy	38° 25′ 09.16″N; 14° 57′ 37.89″E	A	05/06/2013	>500 km	9
Ustica Island, Tyrrhenian Sea, Italy	38° 41′ 32.64″N; 13° 10′ 30.62″E	C	7/27/2011	>600 km	3
S. Caterina, Ionian Sea (Apulia), Italy	40° 07′ 50.86″N; 17° 59′ 39.11″E	C	11/15/2011	>600 km	9
Calvi-Stareso station (Corsica), France	42° 34′ 48.53″N; 8° 43′ 27.57″E	C	10/01/2013	>600 km	4
Blanes (Catalunia), Spain	41° 40′ 43.20″N; 2° 48′ 30.79″E	C	7/27/2012	>1000 km	6
Arcachon, Atlantic, France	44° 39′ 54.05″N; 1° 10′ 42.35″W	C	12/07/2013	Atlantic Ocean	6
Bristol, Atlantic, UK	51° 12′ 50.48″N; 3° 07′ 28.84″W	C	28/07/2011	Atlantic Ocean	15

A acidified sites; *C* control sites

the Mediterranean sites mainly of the brown algae, *Halopteris scoparia*, *Dictyota* spp., *Cladophora* spp. at Ischia, and *Cystoseira compressa* and *Dictyota dichotoma* at Vulcano) and inserting them inside of fabric bags (20 × 20 cm). The bags were then inserted in cool boxes with seawater from the collection site, and transported to the laboratory until sorting of the algae. In the laboratory, the algae were placed into large plastic trays and the worms were visually identified as *Platynereis,* showing a very typical swimming behavior, and gently collected with a pipette and inserted in petri dishes. Worms from each sampling site were fixed in 95% ethanol in separate vials for phylogeographic analysis.

Some of the individuals from the Ischia (S3/S2) and Vulcano (S3) vent's sites were maintained alive after collection and reared under controlled laboratory conditions to check for their reproductive features.

Phylogeographic analysis

The molecular analysis was conducted on specimens collected, as reported in Table 1, and also included the sequences previously published in Calosi et al. (2013) and Lucey et al. (2015). We sequenced a ~600 bp fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) for 3–15 individuals from each population. Total genomic DNA was extracted from each individual worm using the DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) following the manufacturer's instructions. The COI region was amplified using the established primers described by Folmer et al. (1994). PCR products were sequenced directly (Macrogen Europe, Amsterdam, The Netherlands) and the sequence identities were verified using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences were submitted to Genbank under accession numbers KT124668 through KT124717. We further included in our analyses two sequences generated by Lucey et al. (2015), representing a brooding female from the Ischia vents and a free-spawning male from a nearby control site and a COI sequence from the mitochondrial genome of *Platynereis dumerilii* (Boore and Brown 2000) (see Supplementary Information for list of all Genbank accession numbers and haplotype assignment). The sequences were aligned using the ClustalW algorithm in MEGA 7.0.21 (Kumar et al. 2016). MEGA 7.0.21 was also used to calculate average genetic distances among clades using the Kimura-2-Parameter model. The final alignment length was 568 bp. A phylogenetic analysis was conducted using Bayesian Inference in MrBayes 3.2.1 (Ronquist et al. 2012) through the CIPRES Science Gateway v 3.3 (Miller et al. 2010), using two runs with four Metropolis Coupled Markov Chains Monte Carlo (MCMCMC) each for 10,000,000 generations under a General Time Reversible Model plus Gamma, with the first 2,500,000 generations discarded as burn-in. Trees were sampled every 1000 generations from the posterior distribution after the burn-in period and a 50% majority rule consensus tree was generated. *Nereis pelagica* (Genbank accession GU672554) was chosen as the outgroup and *N. zonata* (HQ024403) was included additionally. Minimum Spanning Networks (Bandelt et al. 1999) of the haplotypes were generated in PopART (http://popart.otago.ac.nz). Given the significant genetic divergence

among the major clade, haplotype networks were generated for each clade separately. Genetic diversity indices were calculated in MEGA 7.0.21 (Kumar et al. 2016).

Laboratory rearing and reproductive biology of *Platynereis* vent populations

We kept specimens of *Platynereis* spp. collected both in the vents of Ischia and Vulcano islands, under similar laboratory conditions. The Ischia specimens (Castello Aragonese stations S2/S3) were kept in petri dishes (100 ml, approx. 5 specimens per bowl) using filtered sea water (0.22 µm), and kept in a summer regime of temperature, light and long day photoperiod (21 ± 1 °C and L:D = 16 h:8 h) inside a thermostatic chamber. Chopped fresh spinach was used to feed the worms and specimens checked for water change, food supply, and reproductive status approximately once a week. The specimens collected at the end of May 2015 in the Levante Bay vent's area off Vulcano island were transported to Ischia laboratory alive and reared under the same controlled conditions of the Ischia specimens. For each brooding specimen observed, egg size was measured and larval development was followed and documented by photographs at the stereomicroscope (Leica MZ 125) and optical microscope (Leitz Dialux 20-EB).

Results

Phylogenetic and genetic diversity analysis

The 141 sequences of *Platynereis* spp. grouped into 48 haplotypes (Table 2, Supplementary Table). The phylogenetic tree (Fig. 3) shows that the *Platynereis* spp. sequences of the studied populations form four distinct clades. Clade 1 comprises most of the Ischia vent samples (North and South acidified sites), a few specimens form control areas (San Pietro, Santa Caterina, and Blanes) as well as a confirmed brooding female from the study of Lucey et al. (2015). Clade 1 is formed by three haplotypes, with the dominant haplotype (Hap_01) shared between the Ischia vent samples and one of the individuals from a nearby control site. Clade 2 consists of four haplotypes, mainly of the Vulcano vent samples, plus a single individual from the Ischia vents (S2/S3). Clade 3 includes specimens from three control locations and one heteronereid collected at Ischia swimming in the south vent area. Clade 3 has five haplotypes, with the heteronereid forming its own haplotype. Clade 4 comprises the largest number of sequences (103) and haplotypes (36), all except one (Ischia S3 11) from non-vent sites, and the *P. dumerilii* heteronereids collected at night on the south, acidified areas of the Castello. This last clade also includes the GenBank COI sequence for *P. dumerilii*. The two dominant haplotypes are Hap_15 and Hap_16. Hap_15 is widely distributed throughout the Mediterranean and the Atlantic and also includes one of the heteronereids and the single "outlier" vent sample from the Ischia vents. Hap_16 consists primarily of Mediterranean samples from non-acidified sites and three of the heteronereids. No clear separation between Mediterranean and Atlantic haplotypes is obvious.

Our analyses show that clades 1 and 2 form sister groups, as well as clades 3 and 4. Average genetic distances (Kimura-2-parameter model) are 25.5% between clades 1 and 2 and 22% between clades 3 and 4.

Among the four clades, clade 1 has the highest nucleotide diversity and clade 4 the lowest (Table 1). The Tajima D index is negative in all four clades, with the most negative value in clade 4, indicating that all clades may have gone through recent selective sweeps or population expansions. The starburst shape of the haplotype network of clade 4, with one dominant haplotype and many descendant haplotypes, supports the notion of population expansion. If population genetic indices are calculated by habitat instead of clade, the vent group displays higher nucleotide diversity than the non-vent group. In contrast to all other groupings, the vent population has a positive Tajima D index which may indicate balancing selection and/or a population contraction.

Table 2 Population parameters for vent vs. non-vent populations (excluding the heteronereis) and the four clades of *Platynereis* spp. identified in the phylogenetic analysis

Group	Haplotypes	Samples	P_S	θ	π	D
Vent	7	26	0.2754	0.0722	0.0994	1.4863
Non-vent	41	105	0.2960	0.0566	0.0403	−0.9590
Clade 1	3	20	0.0791	0.0223	0.0147	−1.3588
Clade 2	4	10	0.0525	0.0186	0.0125	−1.5734
Clade 3	5	8	0.0241	0.0009	0.0073	−1.0599
Clade 4	36	103	0.1101	0.0211	0.0066	−2.2000

P_S proportion of polymorphic sites, θ proportion of polymorphic sites to expected number of polymorphic sites, π nucleotide diversity, D Tajima D metric

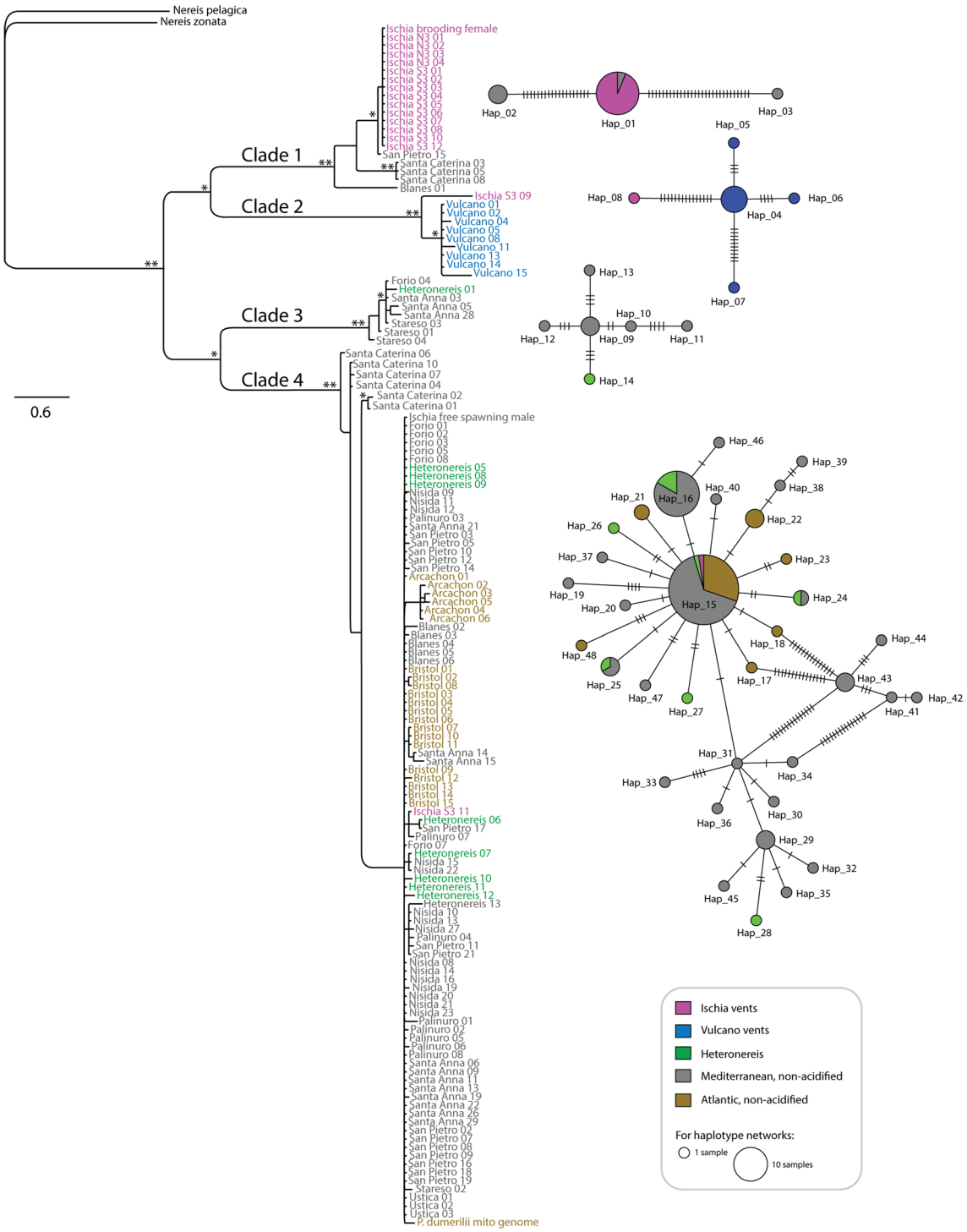

Fig. 3 50% Majority rule consensus tree based on Bayesian Inference. Inference and haplotype networks for each of the four clades. *Asterisks* at nodes indicate posterior probability (*double asterisks* 100%; *asterisks* >90%; branch support values <90% not shown). The size of the *circles* in the haplotype networks indicates the number of sequenced individuals with this haplotype. *Hash marks* on the connecting *lines* indicate the number of mutational steps between two haplotypes. The *colours* identify: Ischia vents specimens in *pink*, Vulcano vents specimens in *blue*, Heteronereis specimens in *green*, and Mediterranean and Atlantic specimens from non-acidified sites in *grey* and *brown*, respectively. Genbank accession numbers and haplotype assignments are listed in Supplementary Table

Observation of reproductive features of *Platynereis* vent populations

Some of the specimens of *Platynereis* collected at the Ischia vent south side area and reared under controlled conditions (see "Materials and methods") laid eggs inside their tubes after a few weeks. Individuals brooding eggs inside their tubes were observed in mid-May 2014 ($n = 3$), mid-June 2014 ($n = 2$), and end of October 2014 ($n = 2$). The eggs measured 250–350 µm in diameter (sample size = 21, sample mean = 308.95 µm; $\sigma = 27.38$) (Fig. 4), according to the embryo developmental stage, and were actively oxygenated through ventilation by regular movement of the parent inside the tube. The large, yolk rich eggs hatched approximately 2 weeks after being laid. Eggs hatched with three-segment juveniles that remained inside the parental tube until they had 5–6 segments (Fig. 4); at 9–10 segments, they started to build their own tubes (Fig. 4). *Platynereis* specimens collected in Vulcano and reared under the same controlled conditions of the Ischia specimens also showed a very similar brooding behavior with deposition of several eggs inside the tube. Specimens with eggs were observed in mid-June 2015 ($n = 8$), mid-July 2015 ($n = 2$), and end of October 2015 ($n = 1$). These parents also were ventilating the eggs, which showed a range size between 250 and 350 µm (sample size = 33, sample mean = 292.21 µm; $\sigma = 26.63$); eggs hatched with three-segment juveniles (Fig. 5), as observed for the Ischia specimens.

Discussion

Our phylogeographic study, in conjunction with reproductive observations on some of the studied populations, reveals several interesting insights. *Platynereis* clades 1 and 2 are primarily comprised of individuals from the vents at Ischia (clade 1) and the Vulcano vent site (clade 2). Both these populations showed brooding behavior, with parental care within the tube and hatching of juveniles form the eggs (Figs. 4, 5). Although most of the sequenced specimens in clades 1 and 2 were sexually immature at the time of preservation, we are confident that clade 1 represents a clade of brooders, because we included the confirmed brooding female from Lucey et al. (2015) in our analyses as a reference specimen. For clade 2, a brooding habit is presumed because of the results from our reproductive studies, which indicated that brooding is the dominant reproductive mode also at the Vulcano vent's site. Additional reproductive

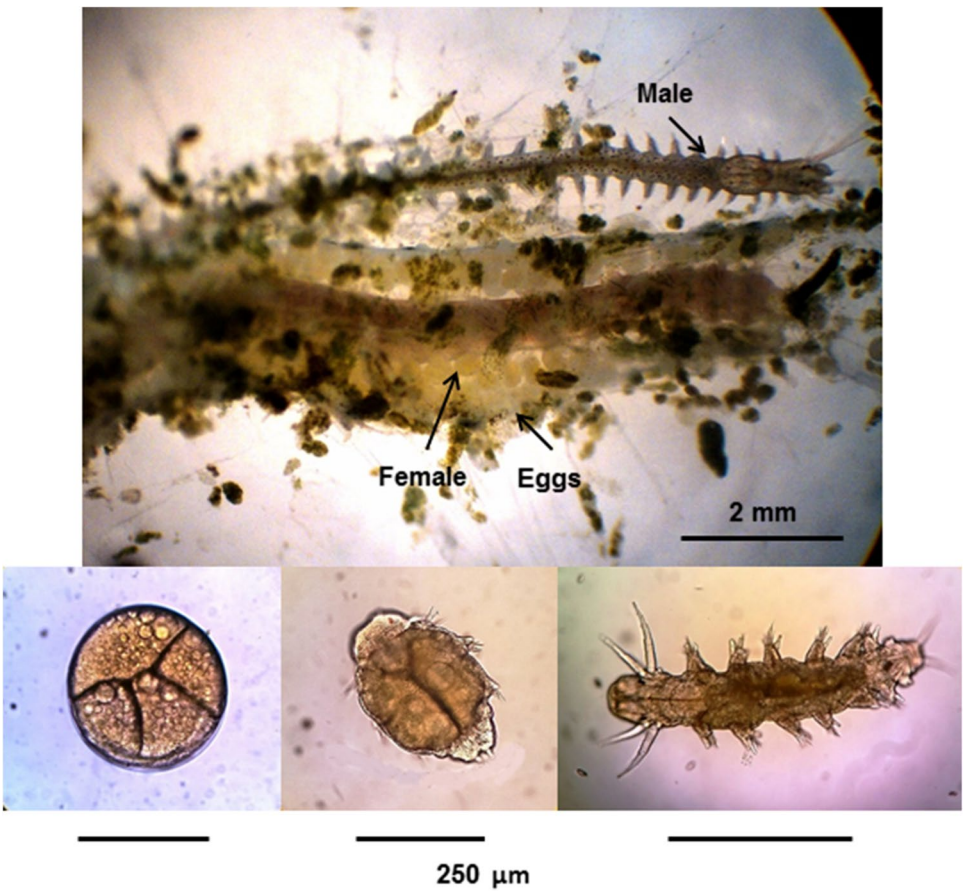

Fig. 4 *Platynereis massiliensis*-like from the Castello Aragonese S3/S2 vent's areas (Ischia island, Italy) with brooding behavior. The pictures depict: the female specimen inside the brooding tube with the laid eggs; the developing embryo inside the egg; a three-segment juvenile rich in yolk; and a six-segment juvenile with some yolk remnants

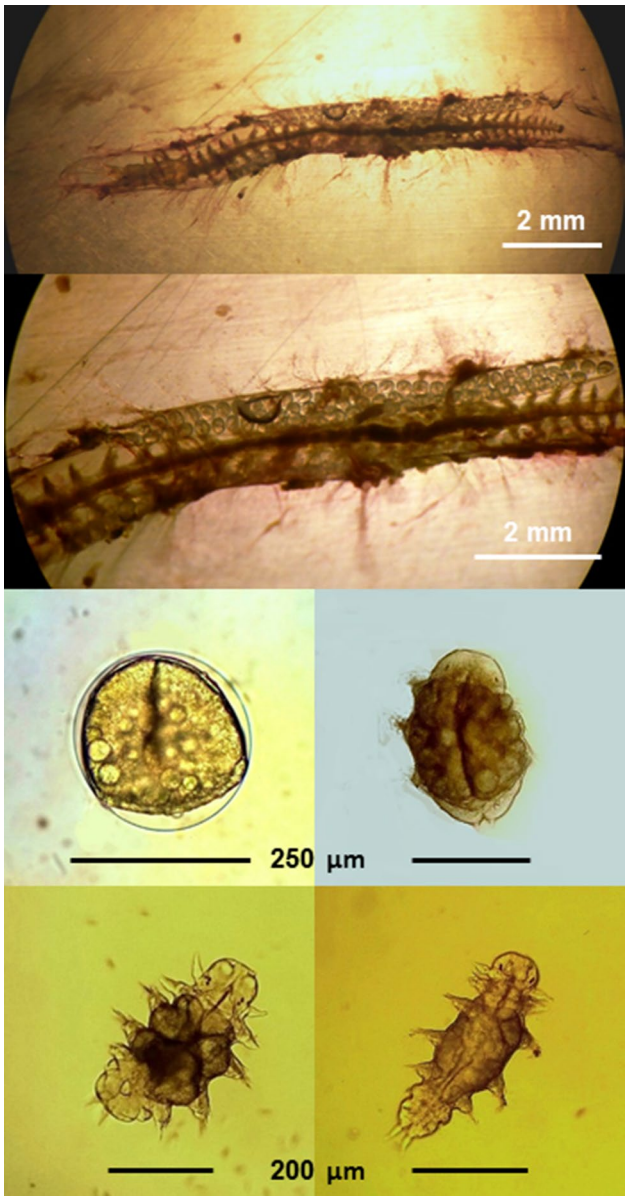

Fig. 5 *Platynereis massiliensis*-like from the S3 vents site off the Levante Bay (Vulcano island, Italy). The pictures depicted: the parent specimen inside the brooding tube, while it is taking care of the laid eggs; a laid egg; three-, four-, and five-segmented juveniles in which the yolk content decreases with body growth

studies with specimens from Vulcano would shed more light onto potential differences between clades 1 and 2.

We cannot conclude with certainty whether clades 1 or 2 represent *Platynereis massiliensis,* because we have not been able to obtain samples from the type locality for this species (off Marseille by Moquin-Tandon in 1869). However, the congruence of our developmental observations with those of Hauenschild (1951) and Schneider et al. (1992), and more recently by Helm et al. (2014), suggests that the Ischia population represents *P. massiliensis*. Hauenschild (1951) collected a population of brooding *Platynereis*, that he named *P. massiliensis*, in the Mergellina harbour (city of Naples) not far from Ischia (approx. 18 nm). Schneider et al. (1992) also recorded a brooding population of *P. massiliensis* in Banyuls sur Mer (France). Despite these studies, *P. massiliensis* was not included in polychaete checklists, including the Italian fauna (Castelli et al. 2008; Mikac 2015), because these previous records were not taken into account in taxonomic/ecological investigations, and ecological surveys, based on the analysis of preserved adult specimens, always reported only *P. dumerilii* (Valvassori et al. 2015). Therefore, we suspect that *P. massiliensis* has been largely overlooked and previous records of *P. dumerilii* should be reconsidered at the light of this "sibling problem". Similarly, based on molecular studies, the occurrence of a complex of species has recently been hypothesized for *P. dumerilii* along the coast of Brazil (Santos C. and Halanych K., pers. comm.).

The significant mean genetic distance in COI between clades 1 and 2 (25.5%) suggests that clade 2 forms another brooding sister species. While genetic distances among sister species in polychaetes can vary greatly, 25.5% is on the high end of the spectrum (Nygren 2014), lending support to the existence of two separate brooding *Platynereis* species. The Ischia and the Vulcano vents populations of putative *P. massiliensis* are more than 600 km apart and such genetic complexity is well known from other nereidid worms that reproduce without epitokous spawning such as in *Neanthes acuminata* (Reish et al. 2014) and in *Hediste diversicolor* (Virgilio et al. 2009). Therefore, comparative sequence data for *P. massiliensis* from its type locality (Marseille, France) would be desirable to verify whether the Ischia or the Vulcano populations (or neither of them) truly represents the originally described *P. massiliensis*.

It was long speculated that the low dispersal rate in many marine species, that have no spawning and no planktonic larval stage, increases genetic diversity (Palumbi 1994) and it is not surprising that a brooding species with a direct or semi-direct development and potentially lower dispersal may show genetically isolated populations. Sato and Masuda (1997) observed two different forms of the nereidid polychaete *Hediste japonica* (Izuka 1908), which differs in life-history strategies (small-egg form and large-egg form). The results demonstrate that the free-swimming larvae of the smaller-eggs form easily migrate, resulting in frequent gene flow among populations; in contrast, the larger eggs form is characterized by direct development into benthic juveniles without a true pelagic phase resulting in limited gene flow between populations (Sato and Tsuchiya 1991; Sato and Masuda 1997). Therefore, the modes of larval development influence the scale of gene flow, consequently affecting genetic differentiation between populations (Sato and Masuda 1997). Low gene flow levels and regional

phylogeographic fragmentation have also been observed by Teske et al. (2007) in two direct developer's marine isopods species and by Reish et al. (2014) in the direct developer polychaete species *Neanthes acuminata*. The *P. massiliensis* genetic complexity (clades 1 and 2) could derive from the reproductive isolation caused by the low dispersal capacity of this semi-direct developer species.

The present data regarding the *Platynereis* spp. population here examined show lineage divergence and presence of putative cryptic species between both types of larval development and potential of larval dispersal. The "free spawner" clade (the *P. dumerilii* complex) has overall lower nucleotide diversity (despite significantly larger sample sizes). The positive Tajima D index of the vent samples (as opposed to negative values for all other groupings) may indicate balancing selection in which a high level of polymorphism within the vent populations may have adaptive advantages. This aspect deserves further study with larger samples sizes collected from multiple venting areas.

A few individuals from non-acidified control populations, including S. Caterina (Eastern Mediterranean), also fall into clade 1. Other individuals from S. Caterina fall into clade 4. This sampling site could represent the geographic area characterized by a species sympatry, in which both putative *P. massiliensis* (clade 1) and P. *dumerilii* (clade 4) coexist.

Clade 4 comprises individuals from non-acidified sites, with one exception (Ischia S2/S3 specimen #11). It further includes the sequence from a confirmed free-spawning male (from Lucey et al. 2015) and the COI sequence from the *P. dumerilii* mitochondrial genome, suggesting that clade 4 represents this species. In our phylogenetic tree (Fig. 3), we treated the *P. dumerilii* mitochondrial genome sequences as originating near the type locality, although the geographic origin of the specimen is actually unknown (Jeff Boore, pers. comm.), and considering the existence of multiple cryptic lineages in *Platynereis*, we cannot exclude the possibility that it was misidentified. However, we have also included sequences from specimens collected in Arcachon on the French Atlantic coast, less than 180 km south of the type locality of *P. dumerilii* in La Rochelle, France, providing that additional support that clade 4 is indeed *P. dumerilii*. Considering that the vents are open systems, it is conceivable that larvae of the epitokous *P. dumerilii* settle in these areas and survive to adulthood. Whether they successfully reproduce under the acidified conditions remains to be fully studied and demonstrated.

Clade 3 is comprised of individuals from several non-acidified sites (including S. Anna, only 600 m from the vent's south side) and one heteronereid from the Ischia vents. The mode of reproduction of these populations has not been studied, but considering that a heteronereid falls into this clade, it appears that they exhibit a similar reproductive mode as clade 4. The significant genetic distance to clade 4 (22%), however, suggests that they represent another sibling species, corresponding in this case to the typical reproductive habit of *P. dumerilii*. From our data, it appears that *P. dumerilii* and *P. massiliensis* represent complexes of sibling species.

The phenomenon of sibling/cryptic speciation is particularly common within the polychaetes even among species used as bioindicators in environmental monitoring (Grassle and Grassle 1976; Durou et al. 2007), or in ecotoxicological and bioaccumulation studies (Virgilio et al. 2005; Burlinson and Lawrence 2007; Vázquez-Núñez et al. 2007; Dean 2008; Blake et al. 2009). There are several species in the cryptic *Perinereis nuntia* group and in the *Marphysa sanguinea* complex that are used in fishing bait trade and correct identification may be crucial for proper management (Glasby and Hsieh 2006; Lewis and Karageorgopoulos 2008). Due to the economic and ecological importance of these polychaetes, proper characterization of local populations is essential to detect potential ongoing cryptic speciation and to evaluate areas of endemism, and thus has fundamental implications for conservation and management (Nygren 2014). One such example of an endangered cryptic species is *Hediste japonica,* whose distribution has been found to diminish at a fast rate (Sato and Nakashima 2003).

The growing availability of DNA sequence data, when combined with more traditional taxonomy based on morphological, life-history and reproductive observations, is leading to an exponential increase in the perception of actual biodiversity (Bickford et al. 2007).

The predominance of the brooding *P. massiliensis* complex in the acidified areas of both Ischia and Vulcano vents might be not directly correlated with the effect of the OA on the reproductive isolation and cryptic speciation, but it seems to be much more related to the success of a brooding habit in stressful conditions. Further experiments to investigate if effectively, the low pH conditions favour a brooding reproductive strategy rather than a broadcasting one would be necessary. In the meantime, the evidence that vent populations only showed a brooding strategy seems to confirm that the extreme conditions in CO_2 vents favour the survival and development of the parental-care taxa rather than free spawners (Lucey et al. 2015; Gambi et al. 2016). Larval stages are often more susceptible to stress than adults and the low pH could represent a sort of barrier for settlement of pelagic larvae coming often from habitat outside the acidified conditions. In contrast, the eggs laying inside a brood tube or egg mass and their successive ventilation and parental care may allow the embryos development until young worms (Schneider et al. 1992). The brooding habit, especially within a tube, might then perform a buffering effect that minimizes the effects of OA by providing a microclimate more favourable for hatching and juvenile development. A further buffer effect of low pH might derive

by the photosynthetic process and oxygen production carried out by algae, where these brooding polychaetes live, thus further facilitating the embryos' survival, as showed also by development of *Spirorbis spirorbis* (Polychaeta, Serpulidae) on the brown alga *Fucus serratus* (Saderne and Wahl, 2012).

Since the extreme conditions of different CO_2 vents systems can be different and may generate different substantial selective pressures besides OA, they can favour cryptic speciation, especially when coupled with the brooding habit of some of the species involved in the selective process. The case of *Platynereis* spp. here discussed prove the occurrence of at least four different species (two complexes of siblings) of which two of them were suspected to belongs to *P. dumerilii* and *P. massiliensis*, respectively. Once the species identity will be resolved, *Platynereis* spp. could represent a good model to study evolutionary implications of climate change environmental stressors on the marine biota, and deserve further analysis in other vent's zones or stressed habitat.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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